Interrogating the Human CD8+ T cell Response in HCV Infection

by

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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

March 2014

Abstract

Hepatitis C virus (HCV) infects nearly 170 million individuals worldwide and remains one of the leading causes of cirrhosis and hepatocellular carcinoma. Approximately 15-20% of individuals will spontaneously clear the infection, with the CD8+ T cell response crucial in achieving control. Given the highly diverse nature of HCV, the T cell response elicited by vaccines against HCV must achieve maximum cross reactivity against widely divergent circulating strains. We assessed T cell recognition of potential hepatitis C virus vaccine sequences generated using three rational approaches: 1) combining epitopes with predicted tight binding to the major histocompatibility complex (MHC), 2) consensus sequence (most common amino acid at each position), and 3) representative ancestral sequence that had been derived using Bayesian phylogenetic tools. No correlation was seen between peptide MHC binding affinity and frequency of recognition as measured by an interferon-gamma T cell response in human leukocyte antigen-matched HCV infected individuals. CD8+ T cells expanded with representative sequence HCV generally more broadly and robustly recognized highly diverse circulating HCV strains than T cell expanded with either consensus sequence or naturally occurring sequence variants. These data support the use of representative sequence in HCV vaccine design. In addition, we hypothesized that ongoing recognition of antigen with continued T cell receptor signaling is associated with upregulation of inhibitory T cell receptors. We characterized evolution of the CD8+ T cell response in the setting of absent, partial or incomplete, and complete recognition of antigen over time to determine if there is an association between prolonged antigenic stimulation and expression of inhibitory receptors. HCV-specific CD8+ T cell from HCV-infected subjects in a prospective longitudinal cohort were phenotyped for the activation markers HLA-DR and CD38; co-inhibitory receptors PD-1, TIM-3 and 2B4; and the memory molecule CD127 using polychromatic flow cytometry. The phenotype of these cells was compared in the setting of intact, mutated, and absent viral antigen within and across subjects. Our data point to the role of ongoing recognition of intact antigen in the T cell response to chronic viral infection

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Acknowledgements

I would like to thank my thesis advisor, Dr. Andrea Cox. It was a leap of faith on both of our part. I had little experience in human immunology work, and was your first graduate student. The conversations that we have had on science, clinical work, and life have been invaluable. I deeply respect your scientific acumen, and it has been a privilege to work with you. In addition, I thank the invaluable contribution of the Viral Hepatitis Lab. I had never seen another lab with expertise across multiple disciplines with such insightful lab meetings. Dr. Stuart Ray has provided invaluable conversations about computer programming and R. The conversations that I have held with the post-doctoral fellows, now faculty (Ashwin Balagopal, Bill Osborn, Michael Chattergoon, and Justin Bailey) have been supremely helpful. The technicians both in our lab as well as the other Infectious Disease labs have provided great assistance and friendship throughout my training.

A huge thank you goes out to the members of the Johns Hopkins Medical Scientist Training Program. In particular, Sharon Welling has been a pillar of support behind the scenes. I also thank the Immunology Training Program and the members of my thesis committee: Robert Siliciano, Drew Pardoll, Chuck Drake, and Jonathan Pevsner. I have talked with each of them both in and outside of committee meetings.

I want to thank all my closest friends from both medical school (Jane Andrews, Audrey Chan) and graduate school (Theodore Kouo, Ali Rabi). My MD/PhD classmates (Erin Parry, Amy Sexauer, Ifeanyi Anidi, and Scott Vandiver) have provided significant emotional support through our years together. I would also like to thank my fiancé, David Liu, for his love and unwavering support. He has seen me when my experiments run late and when I've stressed. He has proofread and discussed my experiments and presentations. He encourages me to be ambitious and to think broadly. He is my best friend, and I look forward to starting the next chapter of our lives together.

Finally, I would like to thank my family: Karly, Mom and Doug, and Dad and Lisa. They have supported me from the very beginning. I love you all.

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Chapter 1

Introduction

This body of work seeks to examine the interplay between hepatitis C virus infection in humans and the resultant CD8+ T cell response. Whereas the majority of humans will progress to chronic infection, a minority will spontaneously clear the infection. The rapid rate of replication and half–life of the virus guarantees that the immune response must defend against an evolving and diverse virus. In general, generating a successful adaptive immune response against a virus, whether through spontaneous clearance of the virus or through vaccination, requires shared responses across hosts. In thinking about this problem in the setting of HCV, I was confronted not only with a genetically diverse virus, but also a cohort of genetically similar-- but not identical-- hosts. I therefore became interested in combining informatics approaches and more classical immunology techniques to study the immune response.

Based on the earlier work in the lab, I had noted that the CD8+ T cell response was heterogeneous across T cell epitopes or within an epitope across time. This insight has led to the applications of technologies to quantify how this T cell heterogeneity impacts sequence selection for a candidate vaccine sequence as well as phenotyping this heterogeneity on a cellular basis. This chapter will orient the reader to the fundamental principles of hepatitis C viral infection, genetic diversity, and the previously described mechanisms by which it is known to evade the immune system. The conclusion will then provide an overview of the hypotheses, aims, and experiments of this research.

Hepatitis C cellular epidemiology

HCV infection is found in virtually every region of the world, and an estimated 170 million persons are infected with HCV worldwide (1). In the United States, there are approximately four million persons with persistent hepatitis C infection and more than 10 thousand HCV-related deaths each year, with mortality from HCV expected to double in this decade and possibly surpass that caused by HIV (2-4). Morbidity and mortality occur in the setting of chronic infection.

Chronic HCV may cause cirrhosis and is the most common indication for liver transplantation in the United States. In addition, HCV is the major cause of hepatocellular carcinoma in the United States, Europe, Japan, Australia, and many other parts of the world (4-8). Although HCV induces both antibody (Ab) and T cell responses, the virus evades them effectively in most cases, with 75% of those exposed becoming chronically infected.

HCV sequence variability

A major challenge in the study of HCV immunology in humans is the high variability of the antigen, which varies not only from person to person, but also at any instant and over time within an infected individual. HCV exists in each infected host as a swarm of genetically-related but distinct variants, collectively called a quasispecies (9-13). This characteristically diverse set of viruses in an individual is not completely random, but rather appears to be driven by the host immune system and balanced by functional constraints (14-17). As a result, each collection of HCV genomes in a quasispecies has a distinctive set of shared characteristics that allow it to be distinguished from others (18). The random generation of sequences results in mutations that may be deleterious or neutral, or offer a selective advantage to the virus because they increase replication efficiency or permit evasion of the immune response.

Hepatitis C cellular immunology

Most viral infections induce successful immune responses and do not persist. HCV has developed mechanisms to evade immune elimination, thereby allowing it to persist in the liver in the majority of infected individuals. The immune correlates that determine whether a patient resolves infection or proceeds to chronic infection are not well defined. Previous studies have shown that spontaneous clearance of HCV infection occurs in association with a broadly specific and vigorous cellular immune response (19-22). In contrast, chronic infection is characterized by low frequencies of specific CD8 T cells in peripheral blood (23-28). Experiments performed with *in vitro* model systems have demonstrated mechanisms by which HCV subverts the innate antiviral responses needed to stimulate lymphocyte effector functions (29-31). This may partially explain

impaired generation of an effective immune response to HCV, but it is unclear how subversion of the innate immune response differs between hosts or how those differences would affect downstream adaptive immune responses.

CD4 T cell responses in humans are more frequently detected and more durable in those who control HCV infection than in those with chronic HCV infection and CD4 T cell responses seen in those who progress to chronic infection have been associated with transient control of HCV RNA (21,22,32,33) . Chimpanzee data support the importance of CD4 T cell responses in control of infection (34). CD8 T cells are also critical to control of HCV and the appearance of HCV-specific CD8 T cells in liver and blood is kinetically associated with control of viremia (35,36). While virus specific CD4 and CD8 T cell responses play a role, generation of a cellular immune response does not ensure control of infection. A detectable cellular immune response is usually present in early infection regardless of outcome and that response may even persist into chronic infection (37). It is unclear why those immune responses fail to control infection, but we and others have demonstrated that the responses generated in acute infection decline in subjects who remained persistently infected (21,36-38). Most subjects with detectable cellular immune responses during the acute phase of infection had gradual loss of responses, in both breadth and magnitude, during the chronic phase of infection. Despite ongoing viremia and ample evidence that HCV sequence varies during acute and chronic infection, those persistently infected did not develop new epitope specificities after the first six months of infection. Taken together, these results suggest that development of HCV-specific T cells is arrested during the first year of chronic infection.

Hepatitis C escape from the immune response

The decline in T cell responses to HCV is poorly understood, but escape is a likely contributing factor. Because immune responses develop over weeks and pathogens replicate on the order of hours or days, it is well-recognized that immune escape mutations may blunt the effectiveness of the immune response (39). Mathematical models of viral kinetics suggest that up to 10^{12} virions are produced each day in a chronically HCV infected human (40). The high level of

virion turnover, coupled with the absence of proofreading by the HCV RNA polymerase, results in frequent mutations within the viral genome. Mutation of class I or II major histocompatibility complex (MHC) restricted T cell epitopes may alter the outcome of infection by preventing or delaying clearance of infected hepatocytes (41). In the face of a vigorous multi-specific cytotoxic lymphocyte (CTL) response, mutation of several epitopes, perhaps simultaneously, would be required for survival of the virus. In the chimpanzee model, antibody-mediated CD4 T cell depletion prior to HCV infection does not prevent initial CD8 T cell responses in a previously-exposed animal, but does impair viral control in association with epitope escape mutations in the viral sequence (34). Longitudinal analysis during chronic infection demonstrated a very low rate of amino acid substitution in CTL epitopes, suggesting that CTL escape that occurs may be limited to early infection (42).

Selection of mutations within T cell epitopes with HCV sequence variation and T cell evasion

To test the hypothesis that immune escape occurs during progression to chronic infection, we compared sites of T cell epitopes with viral amino acid replacements (17). For subjects with persistent viremia and T cell responses, amino acid substitutions were a median of 16.7 fold more likely to occur within T cell epitopes than outside epitopes (range 11.4-24.9). In general, substitutions in recognized epitopes tested resulted in decreased recognition by T lymphocytes compared to recognition of the sequence present at initial viremia, indicating escape. Therefore, virus-specific T cells select for mutants that are not recognized as well by those T cells.

Mechanisms through which mutations permit T cell evasion.

Reduced recognition may result from changes in the epitope sequence itself or in flanking residues that are involved in antigen processing (43). Evasion of the immune response via substitution within T cell epitopes occurs during HCV, HBV, SIV, and HIV infections via two known mechanisms: decreased MHC binding and impaired antigen processing or presentation (17,43-51). Substitutions within T cell epitopes that do not decrease MHC binding or impair

antigen processing or presentation have been observed in multiple chronic viral infections, and substitutions affecting T cell receptor (TCR) engagement have been proposed to explain selection of these mutations. While impaired TCR engagement may allow immune escape, a new T cell repertoire should be available to recognize such neoepitopes. We recently identified a novel mechanism for impaired T cell recognition of a mutation involving a TCR contact residue in HCV (52).

A novel mechanism for impaired T cell recognition of a mutation involving a TCR contact residue

Substitutions in TCR contact residues would be expected to lead to only transient immune escape in immunocompetent hosts since the new epitope should be recognized by a distinct repertoire of T cells specific for the substituted epitope. In addition to identifying multiple mutations which decrease or abrogate HCV peptide binding to the human leukocyte antigen (HLA), we identified an amino acid substitution that reduces T cell recognition due to impaired engagement of the TCR with the variant peptide/A*0201 complex. The mutated peptide was not an antagonist, nor did it affect HLA binding affinity nor prevent antigen processing and presentation. However, it failed to expand T cells primed against the original sequence, poorly activated T cells specific for the original antigen to secrete cytokines, and weakly engaged the TCR when complexed to MHC (52). We determined that the failure to mount a better response to the peptide observed later in infection represented poor intrinsic immunogenicity of this variant because of a 'hole in the repertoire'. This is reflected by an absence or paucity within the primary repertoire of T cells containing high affinity TCR with specificity for that variant. There was a dramatic difference between the ability of the original and variant peptides to prime responses from the primary naïve T cell repertoires in HLA A*0201^{+} individuals never exposed to HCV. The variant virus is highly inefficient at priming naïve T cells specific for that region relative to the virus at initial infection. Thus, in the setting of viral infection, the variant virus can exploit the relative hole in the T cell repertoire as an escape mechanism. This is the first example of a viral escape substitution resulting in evasion of the T cell response via exploitation of a hole in the T cell repertoire.

While substitution within T cell epitopes and the resultant loss of antigen may partially explain the decline of T cell responses generated to HCV in the acute phase of infection with progression to chronic infection, T cell responses to HCV epitopes that do not undergo substitution also decline. Additional evasion mechanisms must be employed to account for the decline observed when escape does not occur and the antigen is preserved.

Mechanisms for evasion of T cell responses other than sequence variation

Detectable levels of CD8 T cell recognition do not always result in escape substitutions and some CD8 T cell epitopes remain intact while others mutate within the same host (17,53). Reversion of immune-escape mutations after transmission to a new host suggests that immune escape can be associated with a significant cost to the virus in terms of fitness (43). It is therefore possible that some substitutions result in virus with such significantly reduced fitness that substitution at that position is not observed. With preservation of viral sequence, alternative mechanisms to evade T cell responses must prevail.

T cell inhibitory receptors

The growing family of inhibitory (or regulatory) receptors may be one of the most important categories of cell membrane receptors participating in the hyporesponsiveness of HCV specific T cells. Although many of these were initially identified on CD4 cells, their expression on CD8 cells has been documented and their engagement appears to inhibit CD8 effector function. One example is programmed death-1 (PD-1). PD-1 is an ITIM-containing inhibitory receptor expressed on activated T cells that binds two known ligands: PDL-1 (also called B7-H1) and PDL-2 (also called B7-DC).(54-57). Anti-B7-H1/PDL-1 and anti-PD-1 antibodies partially restore activity in exhausted LCMV-specific T cells. PD-1 has recently been shown to be upregulated on HCV specific T cells and blockade of signaling through it to enhance their function (58-60). We found that surface PD-1 levels are significantly higher on HCV specific T cells in individuals who fail to control infection versus T cells from those who control infection in both early (<180 days) and later (>180days) infection. Although multivariate statistical analysis indicated PD-1 levels on

HCV specific T cells are directly correlated with HCV RNA levels, high PD-1 levels are also associated with progression to chronic HCV infection *independently of HCV RNA levels* (61). Our comparative analyses of outcome of infection (clearance versus persistence) and PD-1 levels during acute infection demonstrate that PD-1 expression in HCV-specific T cells varies tremendously during acute infection and suggests that it is one of the independent determinants of outcome, a conclusion that bears relevance to intervention with blocking antibodies.

Factors Associated with PD-1 Regulation

In our statistical analysis, we determined that HCV RNA levels and outcome of infection are correlated with PD-1 levels, but not subject gender or age (61). Levels of PD-1 expression can also be dramatically affected by the presence versus absence of pro-inflammatory signals at the time of initial TCR engagement (62,63). In addition to inflammatory stimuli, the presence of antigen is necessary to maintain PD-1 expression. A recent study examining PD-1 expression on CD8 T cells specific from SIV infected macaques demonstrated that PD-1 expression gradually declined on CD8 T cells specific for an SIV-derived epitope that had undergone mutational escape (64). We observed highly variable levels of PD-1 expression on tetramer+ HCV specific T cells during the chronic phase (>180 days infected) in patients who did not clear their infection.

Despite the association between high HCV RNA levels and high PD-1 levels, we observed T cells with low PD-1 levels in the setting of high circulating HCV RNA levels, even at later time points once chronicity has been established. We performed serial viral sequence analysis in subjects and correlated with PD-1 levels on tetramer+ HCV specific CD8 T cells. Viral sequence was assessed at initial infection and at multiple subsequent time points at which T cells specific for known antigens were detectable. Where PD-1 levels decreased despite persistently high HCV RNA levels, we detected substitutions representing T epitope escape mutations in the circulating virus. In contrast, PD-1 levels remained high on virtually all of the T cells specific for HCV epitopes that did not undergo sequence substitution over the period of evaluation. Furthermore, restoration of intact antigen after escape was associated with an increase in PD-1 levels (61). These results provide evidence that immune evasion mechanisms that allow HCV to

persist either involve epitope escape or alternatively, signals that maintain high expression of inhibitory “checkpoint” receptors on virus-specific T cells (Figure 1). Validation of this hypothesis requires defining the functional roles of these inhibitory receptors, which can in part be determined by assessing the effects of *in vivo* antibody blockade of the PD-1 and other inhibitory pathways.

Durability and protective nature of hepatitis C immunity

Although the vast majority of those infected will fail to clear hepatitis C virus, about 20-30% of individuals will clear the virus spontaneously. However, spontaneous control of HCV does not always result in sterilizing immunity. Reinfection with a new strain of HCV is possible and sometimes results in persistent infection. Re-infection provides another opportunity to query parameters of protective immunity. Sequential inoculation of convalescent chimpanzees over a period of 3 years with different HCV strains of proven infectivity resulted in viremic infection with the challenge virus (65). Control of a second HCV infection in chimpanzees that had previously controlled their initial HCV infection was kinetically linked to rapid acquisition of virus-specific cytolytic activity by liver resident CD8 T cells and expansion of memory CD4 and CD8 T cells in blood (35). The importance of memory CD8 T cells in control of HCV infection was confirmed by antibody-mediated depletion of this lymphocyte subset before a third infection. Virus replication was prolonged despite the presence of memory CD4 T cells primed by the two prior infections and was not terminated until HCV-specific CD8 T cells recovered in the liver. The same group also found that memory CD4 T cells are critical to long-term immunity by showing that antibody-mediated depletion of CD4 T cells before re-infection of two immune chimpanzees resulted in persistent, low-level viremia despite functional intra-hepatic memory CD8 T cell responses (34). These experiments demonstrate an essential role for T cells in long-term protection from chronic hepatitis C.

Demonstration of protective immunity following reinfection has been limited within humans due to the low incidence of re-infection and the stringent requirements for longitudinal follow-up. However, a recent study suggests that reinfection with subsequent persistent viremia

may not be uncommon (66). In two epidemiological studies that controlled for infection risk factors, individuals with previous clearance of HCV infection were less likely to develop infection than those infected for the first time and may have a lower risk of acquiring HCV despite ongoing exposure (67,68). Another study found long periods of undetectable viremia in subjects who cleared infection even in the setting of ongoing injection drug use (6). To examine the hypothesis that protective immunity could be achieved in humans, we identified 164 people in the ALIVE cohort (69) who had no evidence of previous HCV infection and 98 individuals who had been previously, but were not currently, infected with HCV(68). We compared the incidence and persistence of HCV viremia in these two groups over four consecutive 6-month periods. Of participants without previous infection, the incidence of HCV infection was 21%. By contrast, people previously infected were half as likely to develop new viremia (12%), even after accounting for risk behavior (hazard ratio, 0.45; 95% CI 0.23–0.88). Furthermore, in HIV-1-negative people who developed detectable viremia, those previously infected were 12 times less likely than people infected for the first time to develop persistent infection (odds ratio 0.05, 95% CI 0.01–0.30), and the median peak HCV RNA concentration was two logs lower. These data suggest that immunity against viral persistence can be acquired, and that vaccines should be tested to reduce the burden of HCV-related liver disease.

We have begun to investigate the cellular and humoral adaptive responses associated with protective immunity through examination of reinfected subjects. A major challenge in the study of HCV immunology in humans is the typically asymptomatic nature of acute HCV infection. We have in part overcome this barrier by longitudinally following injection drug users (IDU's) at risk for infection and with ongoing HCV exposure following control of the initial infection. Sequencing of the virus allows us to identify infection with heterologous virus. These two factors permit identification of members of the cohort who have been re-infected and the opportunity to study the role of the immune system in long-term protection from chronic HCV. Between 1997 and 2007, we identified 113 people who seroconverted with sufficient follow-up to evaluate outcome of infection. Reinfection occurred in 50% of those who controlled their first infection with variable virologic outcomes of subsequent infections. Although viral clearance occurs in

approximately 25% of patients with primary infections, spontaneous viral clearance was observed in 83% of reinfected patients. The incident reinfection rate in our group of cleared seroconverters was very similar to the incident primary infection rate previously reported in the same cohort (70); strongly suggesting that prior clearance of HCV infection does not provide sterilizing immunity against reinfection. However, the clearance rate of reinfection was almost reversed from the clearance rate of primary infection in our cohort, suggesting protection from chronic infection.

Further evidence for the existence of protective immunity against HCV infection comes from analysis of infection kinetics during primary and re- infection. Frequent monitoring of HCV infection status allowed us to assess the kinetics of viremia during initial and subsequent infections within the same subjects. The duration of viremia during primary infection was nearly four-fold longer than in subsequent infections in reinfected individuals. Similarly, the maximum concentration of HCV RNA detected in blood during reinfection was approximately three logs lower compared to initial infection even when persistent reinfections were included in the analysis. The lower duration and magnitude of viremia in the subsequent infections suggest that prior exposure to HCV provides protective immunity against persistent reinfection since fixed genetic factors associated with control of infection would not be expected to improve with each infection.

Humoral immune responses during reinfection

Because the appearance of neutralizing antibodies (nAb) corresponds with clearance of initial infections in some individuals (71), we investigated the role of nAb in control of reinfection. Given the absence of a culture system for circulating HCV strains, the capacity of serum antibodies to broadly neutralize HCV envelope protein binding and cell entry has been assessed using HCV pseudoparticles as previously described (71). During acute infection, nAbs against heterologous viral pseudoparticles were detected in 60% of reinfected subjects. Those nAb drive sequence evolution in the envelope regions of the virus, allowing escape of viral variants that negate neutralization (72). In contrast, cross-reactive nAbs are rarely detected in early infection in patients who progress to chronic infection (71). Compared with the initial infection, HCV

reinfection is associated with a reduction in the magnitude and duration of viremia, broadened cellular immune responses, and the generation of cross-reactive humoral responses. These findings are consistent with the development of adaptive immunity that is not sterilizing but protects against chronic disease.

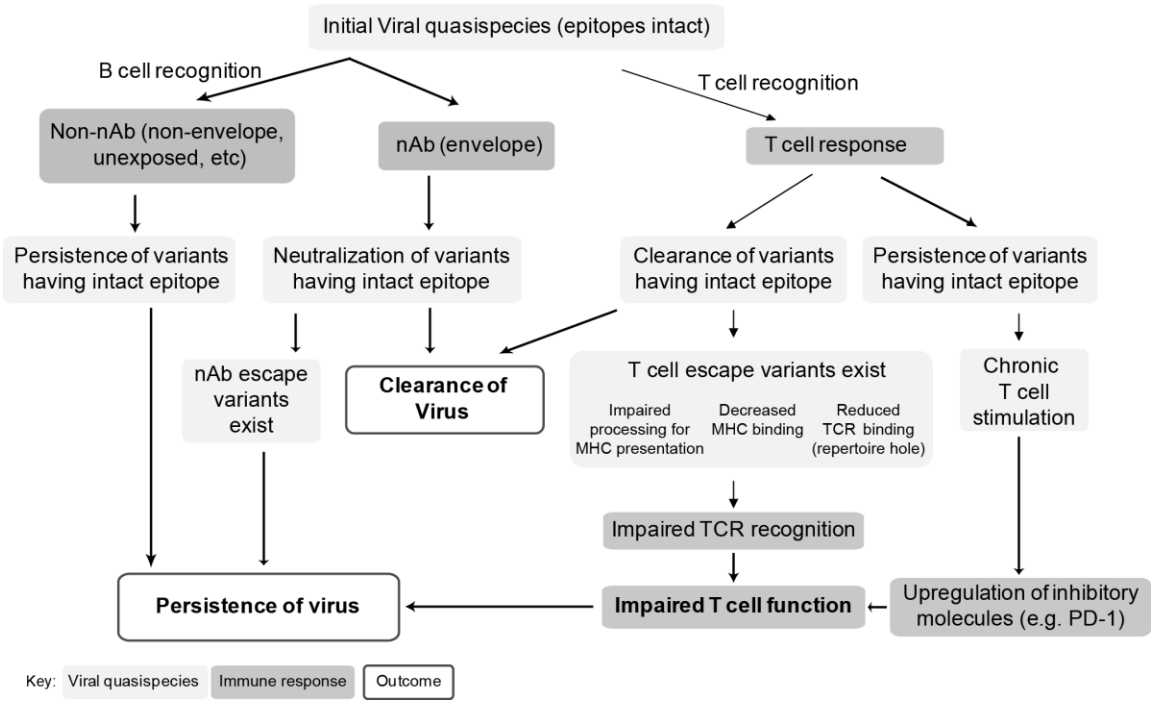


Figure 1 Viral evasion from the adaptive immune response

The constraints placed on the virus due to immune pressure lead to clearance or select for mutations that successfully evade the immune response. nAb = neutralizing antibody. TCR = T cell receptor.

Dissertation Overview:

Overall goal:

Dissertation hypothesis: To investigate mechanisms through which HCV specific CD8+ T cell function is maximized, both through vaccine induction of responses and through understanding the relationship between sequence diversity and T cell regulation (activation and inhibition). In order to optimize T cell based vaccines against HCV, it is crucial to 1) select vaccine antigen that maximizes T cell function and cross reactivity and 2) reduce upregulation of T cell inhibitory molecules and promote effective memory T cell production.

After writing a review of the mechanisms through which chronic HCV evades immune clearance, I focused on selection of vaccine antigens that enhance expansion of functional CD8 T cells against diverse HCV sequences. We suggest that the optimal CD8+ T response against HCV responds to a phylogenetically rooted ancestor. Mutations away from this sequence result in decreased or absent recognition as measured by cytokine functionality or surface receptor expression patterns. However, mutations that revert to the original sequence or sequences that structurally bind similarly to the HLA receptor will result in restoration of the optimal sequence recognition.I then sought to characterize the evolution of the CD8+ T cell response in the setting of absent, partial or incomplete, and complete recognition of HCV antigen. We have hypothesized that ongoing recognition of HCV antigen acts on the level of T cell receptor.

Specific Aim 1:

To determine whether a rational or computational approach to generate a T cell based vaccine would maximize T cell responses

Approach: We assessed T cell recognition of potential hepatitis C virus vaccine sequences generated using three rational approaches: 1) combining epitopes with predicted tight binding to the major histocompatibility complex (MHC), 2) consensus sequence (most common amino acid at each position), and 3) representative ancestral sequence that had been derived using Bayesian phylogenetic tools.

Specific Aim 2:

To determine the relationship between HCV-viral sequence changes and CD8+ T cell expression of co-inhibitory and activation surface molecules.

Approach:

CD8+ T cells were phenotyped using polychromatic flow cyometry to measure simultaneously the presence of activation and co-inhibitory molecules. Longitudinal viral sequence analysis was also performed to identify the circulating viral sequence prior to the onset of the adaptive immune response and throughout the course of infection after induction of T cell responses. The T cell phenotype of was then correlated with antigen status; intact, mutated, or absent.

Chapter 2

Introduction

In the United States, hepatitis C virus infects 4 million individuals and remains the leading cause of liver transplantation and hepatocellular carcinoma (2,3). The estimated morbidity associated with infection is anticipated to rise over the coming decade. Recent pharmacological advances promise interferon-free, direct acting anti-viral regimens with >90% achieving a sustained virological response and set to be licensed in the coming years (73-76). However, such treatment carries a high price tag, does not completely reverse HCV associated liver disease, and does not prevent reinfection. Thus, development of an HCV vaccine to prevent HCV infection remains a critical public health need despite the fact that no vaccine is currently available.

Due in part to its highly error-prone NS5B polymerase, HCV circulates within and between individuals as a quasispecies (13). This swarm of viruses presents an immense challenge for vaccine development. Vaccine strategies meant to overcome this viral diversity must generate a broad immune response, capable of responding to a host of variations. Initial efforts at vaccine development focused on development of sterilizing immunity using an E1/E2 heterodimer (77). Neutralizing antibodies against envelope can play a role in clearance of infection and re-infection (71,78,79). However, specific envelope sequences that drive broadly neutralizing antibody production and sterilizing immunity remain unknown. Generation of robust CD4+ and CD8+ T cell responses is considered critical for long-term immunity (19-21). Development of a cytotoxic T lymphocyte (CTL) response usually occurs early in infection and has a kinetic association with clearance of viremia (35). Failure of a CTL response to control viremia may be due to evasion of the T cell response through a number of mechanisms, including lack of CD4+ T cell help(41,80), T cell exhaustion(58,61,81,82), or the emergence of viral escape mutations (17,43,44,47,48,50,83). Although the cellular immune response to HCV declines in chronic infection, progressive broadening of the T cell response to HCV is associated with enhanced control of HCV upon repeated reinfection (78). The generation of cellular immunity

capable of controlling HCV infection analogous to that observed in people who successfully control repeated HCV infections has become a goal of HCV vaccine development(84).

The T cell responses generated by a vaccine must provide cross-reactivity against highly diverse circulating strains, making selection of HCV vaccine antigens a challenge. Sequence strain selection to induce a T cell response, either for peptide or DNA-based vaccines, has borrowed heavily from HIV vaccine design (85,86). Strategies that deliver only a handful of epitopes or provide cross-coverage of multiple epitopes of a single HCV-protein may maximize recognition of that protein, but at the expense of other regions that may be important in protective immunity. Another strategy that has been shown to be effective for generating cellular immune responses using hepatitis B, lymphocytic choriomeningitis, and lassa virus vaccines in mice is selection of peptides with known high affinity for the major histocompatibility complex (MHC) (87-89). High affinity binders may serve as a stronger immunogenic agent and peptides with high MHC affinity have been shown to be recognized by HBV and HIV infected humans and SIV infected macaques (90-92). However, the frequency of recognition of epitopes selected on the basis of MHC affinity for HCV is unknown.

An alternative to the inclusion of specific epitopes that is commonly employed in vaccine strain selection is the use of a single circulating strain. The choice of which viral strain to use is arbitrary given that no circulating strain resembles the majority of other circulating strains of HCV. The extent to which any individual strain might have mutations that impair immune recognition and allow persistence is also unknown. Strain selection for HCV has focused on existing, well-characterized strains, such as the genotype 1a strain H77 (93). HCV strains between subtypes can differ in nucleotide composition by 20-25% (94). By comparison, a less than 2% amino acid difference can cause a failure in cross-reactivity of the polyclonal response to influenza vaccine (85). An alternative to selection of specific epitopes or use of a single circulating variant is to use synthetic, computer-generated strains to minimize the degree of sequence dissimilarity between a vaccine strain and contemporary circulating viruses. There are multiple computational methods for generating a vaccine strain that minimizes the amount of sequence divergence (distance) between the antigen and contemporaneously circulating viruses. One method for artificial

sequence design is to create a consensus strain, whereby the most commonly predicted amino acid at each position is used at each position (85). A limitation of the consensus approach is that escape mutations can become the dominant sequence type in regions where the restricting HLA allele is common, as recently demonstrated for HCV (95). In contrast, phylogenetic reconstruction can distinguish residues with shared ancestry from those selected by individual hosts (96). We recently generated a representative synthetic HCV clone (bole1a) using Bayesian phylogenetic methods (97). This approach allowed calculation of the most probable base at each position using 390 HCV-1a sequences and an outgroup of HCV-1b sequences. While not naturally occurring, this strain's sequence is similar to currently circulating HCV strains and lacks the divergent escape mutations carried by circulating strains. Therefore, bole1a may have enhanced ability to elicit robust, cross-reactive responses. However, the enhanced potential of any computer generated sequence to elicit cross reactive T cell responses has not been demonstrated for HCV.

We assessed the recognition frequency and cross reactivity of HCV sequence peptides generated using the three vaccine development methods described: peptide selection based on high MHC binding capacity, selection of a single circulating strain, or use of the algorithmically derived representative bole1a sequence. We provide the first data in HCV to support the use of a synthetically generated sequence to elicit robust CD8+ T cell responses.

Methods

Subjects

The Baltimore Before and After Acute Study of Hepatitis (BBAASH) cohort of injection drug users (IDU) is a prospective study of injection drug users at risk for hepatitis C infection. Eligible participants have a history of or ongoing intravenous drug use and are seronegative for anti-HCV antibodies at enrollment. Written consent was obtained from each participant. Once enrolled, participants receive counseling to reduce intravenous drug use and its complications (17). Blood is drawn for isolation of serum, plasma, and peripheral blood mononuclear cells (PBMC) in a protocol designed for monthly follow-up. Participants with acute HCV infection were

referred for evaluation of treatment. The study was approved by the Institutional Review Board at the Johns Hopkins School of Medicine.

Hemigenomic HCV sequencing:

Total RNA was extracted from serum or plasma using the QIAmp viral RNA mini kit according to manufacturer's directions (Qiagen). A 5.2kb region spanning the 5-untranslated region to the NS3/NS4A junction was reverse transcribed, amplified by nested PCR, and cloned as described previously(98). Briefly, total RNA was extracted from serum using the QIAamp viral RNA mini column (Qiagen) according to manufacturer's instructions. The 5.2 kb PCR product was generated by a reverse transcription nested PCR strategy. Purified PCR products were cloned into a pCR-XL TOPO vector and transformed into One Shot TOP10 chemically competent cells. Forty clones were picked and amplified with Templiphi to screen for the presence of the insert. Positive clones were sequenced and aligned using CodonCode Aligner (Codon Code Corporation, Dedham, MA).

Human Leukocyte (HLA) binding assay:

Quantitative assays to measure the binding of peptides to purified class I molecules were based on the inhibition of binding of a radiolabeled standard peptide (99). Briefly, 1-10 nM of radiolabeled peptide was co-incubated at room temperature with 1 μM to 1 nM of purified MHC in the presence of 1 μM human β_2-microglubulin (Scripps Laboratories, San Diego, CA) and a cocktail of protease inhibitors. After a two-day incubation, binding of the radiolabeled peptide to the corresponding MHC class I molecule was determined by capturing MHC/peptide complexes on Greiner Lumitrac 600 microplates (Greiner Bio-one, Longwood, FL) coated with the W6/32 antibody, and measuring bound cpm using the TopCount microscintillation counter (Packard Instrument Co.). Alternatively, following the two-day incubation, the percent of MHC bound radioactivity was determined by size exclusion gel filtration chromatography using a TSK 2000 column.

Generation of HCV genotype 1a consensus sequence (cons1a)

Full-length genotype 1a polypeptide sequences (n=390) were downloaded from GenBank that were circulating in humans and were non-artificial sequences. Sequences were aligned to H77 and the consensus residues calculated with MargFreq program.

Generation of HCV genotype 1a sequence (bole1a)

Construction of the bole1a sequence has been described elsewhere (97). Briefly, the bole1a sequence was constructed using Bayesian phylogenetic and ancestral sequence reconstruction methods along with covariation analysis on the same set of 390 full-length sequences from which the consensus was derived.

Cell culture to generate T cell lines:

PMBCs were thawed and stimulated with 10ug/ml of synthetic peptide and 0.5ug/ml of anti-CD28 and anti-CD49d antibodies. Cells were maintained at a density of 2 x 10^6 cells/ml in RPMI 1640 [Sigma-Aldrich], 20% Human serum [Sigma-Aldrich], and 10mM HEPES buffer [Sigma-Aldrich] with 2 mM glutamine and 50 U/ml penicillin-streptomycin. Cells were incubated at 37ºC and 5% CO2 with addition of rIL-2 on days 3, 4, 7, and 10 and again following a second round of peptide stimulation on day 10.

Selection of epitope variant peptides and peptide synthesis:

In subjects with detectable CD8+ T cell ELISpot responses against HCV, we examined the sequences of the epitope regions over time. When changes away from the consensus sequence occurred in the region of a CD8 T cell epitope, a synthetic peptides corresponding to that sequence as well as the consensus sequence were synthesized commercially by Genemed Synthesis (San Antonio, TX).

Interferon-gamma ELISpot Assay

Ex vivo HCV CD8+ T cell responses were quantified by Human IFN gamma ELISPOT Ready-SET-Go!® assays (eBiosciences) according to manufacturer's instructions. Briefly, PBMC were screened for recognition of HCV-specific antigens using pools of overlapping peptides covering the entire HCV polyprotein and previously defined optimal epitopes. Once responses were determined, additional analyses were performed using decreasing concentrations of synthesized peptides to determine the effects of amino acid substitutions on recognition and the degree to which there was cross-reactivity with circulating variants of the epitope and consensus sequence peptides

To compare the magnitude of T cell responses generated in response to optimal and variant peptides, the same ELISpot assay was performed, using decreasing concentrations of synthesized peptides as antigen. Briefly, PVDF plates were coated with 2.5ug/ml recombinant human anti-IFN-gamma antibody (Endogen M-700A) in 100ul PBS/well at 37ºC at 4ºC overnight. Plates were washed with sterile PBS eight times before blocking with RPMI +10% FCS for 30 minutes. Either 20,000 or 30,000 cells in R10 media were added to the wells. Decreasing peptide concentrations (10ul/well, 10ug/ml-0.001ug/ml) were added to the well in duplicate. PHA served as a positive control. Plates were incubated for 20 hours at 37ºC with 5%CO2. Following incubation, plates were washed with 200ul sterile PBS eight times and blotted dry. Biotin-labeled anti-IFN-gamma (Endogen, M-701B, 0.25ug/ml, 100ul) was added to each well and incubated for 90 min at room temperature. Plates were washed and incubated with streptavidin-alkaline phosphatase (Bio-Rad 170-3554, 100ul) for 45 minutes at room temperature. Following additional washes, the plates were developed with BCIP/NBT Tris-buffer (pH 9.5) solution (Bio-Rad, 170-6532 and 170-6539) according to manual instructions. Plates were dried overnight and read on a Ziess ELISpot plate reader.

Statistical Analysis

Statistical analysis was done using SigmaPlot software version 12.0 (Systat Software, Inc.). Log odds of frequency of recognition based on HLA matched subjects was calculated. Linear regression was performed on the log odds versus log of the IC50 from the in vitro binding

assay for HLA-matched sequences for positive responses in all subjects and among those that cleared acute infection.

Results

HLA binding affinity does not predict frequency of recognition.

Peptides that bind strongly to the MHC can induce strong pathogen -specific immune responses in other chronic viral infections (90-92). As a result, selection of pathogen peptides with high MHC binding capacity has been used as a strategy in vaccine strain selection. In order to test the hypothesis that high affinity binding to Class I MHC is associated with increased frequency of HCV peptide recognition, the likelihood of recognition of previously identified class I restricted epitopes was correlated with the IC50 binding to its corresponding HLA allele and subtype. Our cohort is routinely screened during acute infection for responses against overlapping genotype 1a peptides, including both long regions of the HCV polypeptide and known class I epitopes (17). Assessment during the acute phase of HCV infection is critical since the breadth of HCV epitope recognition declines with progression to chronic infection (20,37).

A total of 60 acutely HCV infected subjects had HLA genotype data available and were screened for HCV specific T cell responses to 35 epitopes with known HLA restriction and binding affinity (Table 1). To avoid spuriously negative results, the calculated frequency of responses against each known HLA-restricted epitope in the ELISpot assay includes data for only those subjects with matching HLA genotype and subtype. A positive response for a subject to any epitope was defined as recognition of that epitope in ELISpot testing at any time during infection. The frequency of recognition varied widely between epitopes. For example, we found that nearly half of HLA-A*0201 individuals (14/33, 42%) recognized the HLA-A*02-restricted epitope NS3 1406-1415 (KLVALGINAV) (Table 1). In contrast, 13/35 (37%) of the tested epitopes were never recognized despite being restricted to 23 different HLA subtypes present in 54 different subjects tested. To determine if recognition frequency was correlated with HLA-binding affinity, affinity and recognition frequency were compared.

An *in vitro* competitive binding assay of peptide to the restricting HLA allele was used to assess the strength of binding to the MHC (Table 1), with some of these results reported previously (100,101). This assay measures HLA binding affinity of test peptides by determining the concentration of test peptide required to inhibit by 50% binding of radiolabeled peptides to purified HLA molecules of known subtypes. Requiring high concentrations of test peptide to compete with binding of the radiolabeled peptide to the restricting MHC (high IC50) suggests low affinity for that HLA molecule. We compared the frequency of epitope recognition among HLA-matched individuals to the inhibitory concentration in the *in vitro* HLA binding assay, and found no significant relationship (Figure 2A). For all subjects and responses, HCV peptides with higher HLA binding affinity were no more likely to be recognized than those with low binding affinity (y=-0.1254x-0.4992, r^2=0.0492, OR=0.882, 95%CI 0.69-1.12). Moreover, 12/19 (63%) high affinity HCV peptides (IC50 <50nM) were never recognized at any time point examined in our large cohort of acutely HCV infected subjects. This lack of relationship between frequency of recognition and HLA binding affinity held even when we examined the subset of subjects who cleared acute infection and therefore had successful immune responses (Figure 2B, y=-0.1464x-0.2524, r2=-0.0925, OR=0.866, 95% CI=-13.0-12.7). Having found no association between HLA binding affinity and T cell recognition, we evaluated immunogenicity of vaccine strains generated in alternative ways.

Bole1a sequence induces more robust responses than consensus sequence.

The use of consensus or representative sequences has been proposed in vaccine design to minimize the genetic differences between vaccine strains and contemporary isolates. A consensus sequence includes the most common amino acid at each position. However, such a sequence is subject to the frequencies of common HLA alleles in the population and the forces by which these alleles shape circulating viral sequence. Evolutionary forces on the population level due to common HLA alleles play a contributory role on the frequency of circulating escape variants (95,96). The methods used to generate bole1a minimized the genetic distance from circulating sequences while maximizing the likelihood that selected residues represented

universally-shared (i.e. rather than individual) evolutionary forces. For comparison, we used the same 390 full length genotype 1a HCV polypeptide sequences that were used to construct bole1a to generate a consensus sequence (cons1a). Bole1a sequence contains a larger number of known T cell epitopes than do the H77 and HCV-1 strains (97) despite H77 and HCV-1 having been used widely to identify HCV epitopes. Bole1a is less likely to contain escape mutations that would impair T cell recognition (85). The cons1a and bole1a sequences were compared for homology across 15 epitopes located between Core and NS3. For 13/15 epitopes, the sequences were identical with the two exceptions noted in Table 2. In general, the consensus amino acid residue was clearly defined, with 90% or higher frequency of a single residue. Where the consensus amino acid differed from bole1a, the frequency at the differing position was between 0.405 and 0.523.

T cell recognition of artificial HCV sequences has not yet been demonstrated. Therefore, we initially sought to determine if the artificially generated HCV vaccine sequences cons1a and bole1a could induce robust T cell responses and if the sequences differed in their capacity to do so. The cons1a and bole1a sequences were tested for their capacity to expand T cells already primed *in vivo* against circulating HCV. Lines were generated against peptides encoding the two cons1a and bole1a sequences that differed and then tested for recognition of both the cons1a and bole1a sequences by IFN-gamma ELISpot (Figure 3A and 3B). In both cases, the bole1a sequence peptides were able to expand T cell responses that recognized bole1a and consensus sequences for that epitope. In contrast, incubation with the consensus sequence expanded T cells well for only one of the two epitopes. Expansion with consensus NS3 $_{1436\text{-}1444}$ (ATDALMTGF) resulted in minimal recognition of either variant of that epitope (Figure 3A). Expansion with consensus $E2_{610\text{-}618}$ (HYPYRLWHY) recognition of both variants, but of much lower magnitude than that achieved with bole1a sequence expansion (Figure 3B). Overall, T cells expanded with the bole1a sequence-encoded peptides had greater magnitude responses against both bole1a and consensus sequences than those sequences expanded with cons1a (Figure 3C). Given the improved expansion using bole1a compared to cons1a, future comparisons of the

immunogenicity of artificial strains to circulating HCV strains were completed using bole1a sequence for these epitopes.

Bole1a sequence better expands CD8+ T cells of diverse HCV specificity

Mutation within CD8+ T cell epitopes occurs frequently, with 69% of epitopes undergoing mutation in the first six months of infection(17). We and others have previously demonstrated selection of and subsequent dominance of HCV variants that evade neutralizing antibody and CD8+ T cells responses (17,71,102). Thus, any given circulating HCV strain from an individual is likely to contain a large number of escape mutations that allow evasion of T cell recognition (44,103). The capacity of a vaccine strain to induce CD8+ T cell responses to naturally occurring sequence variants will likely be reduced if the vaccine strain used contains escape mutations.

Naturally occurring HCV variants within epitope regions were identified through longitudinal hemigenomic sequencing of HCV from subjects (Table 4) who recognized CD8+ T cell epitopes of known HLA-restriction. Identified amino acid substitutions within these epitopes are listed in Table 3. Bole1a and consensus sequences were identified in circulating sequences for all epitopes investigated, confirming that sequences algorithmically chosen are also found in natural infection.

Given that representative HCV strains should contain fewer escape mutations than do naturally circulating strains, we hypothesized that CD8+ T cells already primed *in vivo* against HCV would better recognize bole1a sequence than any individual circulating strain. Expansion capacity of the bole1a sequence relative to naturally occurring sequence variants was tested. Lines were generated against peptides encoding the bole1a sequences or naturally circulating sequence variants. These lines were tested for recognition of the bole1a sequence and the corresponding naturally occurring sequence variants using peptide dilutions in an IFN-gamma ELISpot assay (Figure 4A-G). Lines were successfully generated against 8/8 (100%) peptides encoding bole1a sequences and for 12/18 (67%) peptides derived from naturally occurring sequence variants that did not match bole1a (Figure 4A-G and Figure 3A and B). Incubation of T cells with every naturally occurring sequence variant of the $Core_{41-49}$ epitope (Figure 4A) as well

as three of the four $NS3_{1073-1081}$ (Figure 4F) epitopes failed to generate any ELISpot responses above background to bole1a or sequence variant antigen peptides. This suggests that the naturally occurring variants of these epitopes are less able than bole1a to expand cross reactive T cell responses. In addition, when expansion of T cells with naturally occurring sequence variants did occur, the responses were of diminished magnitude relative to those obtained using the bole1a sequence to expand. The single exception occurred for $p7_{790-790}$, where expansion with either the bole1a sequence or a naturally occurring sequence variant produced comparable (within 2-fold) recognition of both sequences across four of five peptide dilutions (Figure 4E). Figure 4H shows the summation of ELISpot responses against 10, 1, and 0.1uM of the bole1a and every variant peptide for each epitope when bole1a is used to expand (left column) or a naturally occurring variant is used to expand (right column). For 6/7 (86%) epitopes, expansion with bole1a sequence resulted in stronger summed responses against itself and sequence variants as measured by total SFC per million cells than did expansion with circulating variants (Figure 4H). The single exception, the $p7_{790-790}$ sequence, still resulted in an overall strong magnitude of response against both the bole1a sequence as well as the naturally occurring sequence variant. Thus, bole1a best or comparably expands T cells specific for HCV epitopes, again supporting the superiority of the bole1a sequence over circulating variants in expanding T cell responses broadly specific for HCV.

Bole1a sequence expands T cells of diverse cross-reactivity.

The level of cross-reactivity for seven epitopes was compared across individuals to determine if recognition patterns were consistent across subjects and if bole1a expanded T cells specific for variants not seen in that subject. We tested our CD8+ T cell lines against the indicated bole1a sequence for recognition of all the epitope variant peptides identified in our sequencing of circulating HCV strains as well as bole1a and consensus sequence epitopes (Figure 5A-G, Table 3, Table 5). The bole1a sequence was recognized by all subjects and for every epitope at least the two highest concentrations of peptide. However, the recognition patterns for specific variants did vary between subjects and by epitope. For example, expansion

of T cells with either the peptide encoding bole1a sequence for the epitope $NS3_{1436-1444}$ or its variant resulted in similar recognition patterns of bole1a and the variant sequence (Figure 5A) between subjects. In other cases, expansion with bole1a-encoded sequence resulted in better recognition of the bole1a sequence than any variant, but with differing recognition profiles of variant sequences (5B-D). Finally, expansion with bole1a-encoded sequence resulted in equal recognition of sequence variants and itself for three epitopes (Figure 5E-G), although the extent of variant recognition differed across subjects. T cells expanded from both subjects recognized the D610H and D610N variants well with diminished recognition of the other three sequence variants (Figure 5E). These results suggest that expansion of T cells with bole1a is likely to generate T cell responses that recognize many different circulating HCV variants in different HCV exposed subjects. In contrast, expansion with any given circulating variant results in widely different levels of recognition depending on the subject and HCV exposure.

Bole1a sequence contains the greatest number of optimal epitopes.

Following vaccination against any virus, individuals subsequently exposed to that virus will have to respond to a multitude of sequences similar but not identical to those they have already seen. Optimal sequences, or those sequences that stand the greatest likelihood of being cross-reactive in a vaccine, were defined as sequences that a) induce a response when used to generate a line and b) induce maximal responses. For a given epitope position, the optimal sequence was defined experimentally as the sequence that induced the greatest recognition on IFN-gamma ELISpot at a peptide concentration of 1uM. Lines generated that produced only responses less than 100 SFC/1E6 cells were excluded from consideration. Of the ten epitopes assessed, six had a single optimal sequence. For four epitopes, two sequences were equally well recognized with responses of a magnitude within two-fold of the strongest response. When two sequences produced comparable responses, both were deemed optimal. For example, the WPAPQGARSL and WPAPQGSRSL variants of the $NS3_{1111-1120}$ epitope were comparably recognized in multiple ELISpot assays across multiple individuals so both were accepted as optimal sequences.

The presence of the optimal sequence or sequences for the ten epitopes tested was assessed across the 390 full length genotype 1a sequence culled from GenBank as well as the bole1a and cons1a sequences. The tabulated data are presented in Figure 6. Bole1a was found in the group of sequences containing the highest number of optimal epitopes and contains optimal sequence for every of the ten epitopes. Twenty nine other viral strains also contained all of the optimal sequences. In comparison, the cons1a and the H77 sequence were found to contain only 9/10 optimal epitopes despite the H77 strain being used to define most of the known HCV epitopes.

Discussion

Successful vaccination strategies against extremely genetically diverse viruses like HCV will require generation of an immune response that is both broadly cross-reactive and robust against the antigens to which vaccine recipients are subsequently exposed. Significant controversy exists about the best method for selection of a vaccine strain capable of inducing strong, cross-reactive T cell responses against divergent circulating strains. Clinical trials in humans comparing the capacity of different vaccines to induce protective responses are not possible due to expense and limited access to those at risk for infection. Thus, surrogate measures for vaccine strain efficacy are needed. We elected to focus on HCV genotype 1a infectious strains as the most common infectious subtype in the US as well as one of the most difficult to eradicate by therapy. This study presents a comprehensive analysis of the efficacy of a computationally designed genotype 1a HCV sequence (bole1a) to expand HCV specific T cell responses relative to a consensus sequence or individual circulating strains as well as an assessment of the relationship between HLA binding affinity and recognition.

Beginning with the method of selecting peptides with high HLA binding affinity as vaccine antigens, we assessed the frequency of peptide recognition relative to HLA binding capacity. If peptides bound more strongly to the MHC induce recognition more frequently, those peptides might be better candidates for a vaccine sequence. For hepatitis B, lymphocytic choriomeningitis, and HIV, high affinity peptides have been proposed as vaccine antigens based on their enhanced

immunogenicity (87,89,91). However, we found no association between HCV peptide binding to the MHC and frequency of recognition in an acutely infected HCV cohort. The lack of association also held among those who clear infection, suggesting that the strength of binding to the MHC does not predict likelihood of developing an effective HCV response. High HLA binding affinity as a method for producing antigen has been proposed in previous studies (100), (101). To evaluate this strategy, a previous study assessed the capacity of peptide with high HLA A*02 binding affinity to prime naïve T cell responses *in vitro* using human cells and *in vivo* in mice. While some peptides were capable of inducing responses in both models, it remained unclear from that study if *in vivo* priming of HCV specific T cells favors recognition of high affinity HLA binders (100). Our study compares *in vivo* human recognition of peptide epitopes restricted to multiple HLA alleles with widely diverse binding capacity. Another study proposed the use of high HLA binding affinity with ability to bind multiple alleles to select HCV vaccine antigen and found that peptides with comparable HLA binding capacity could have markedly different immunogenicity (101). This is supportive of our study's finding that HLA affinity beyond that needed to bind was not the major determinant of recognition. Once MHC binding beyond a relevant threshold is achieved, other factors such as location of the epitope in the polypeptide, its abundance, thymic education, and T cell precursor frequency appear to play a dominant role in selecting the actual epitope recognized (104,105).

The most commonly proposed method in vaccine design for HCV is selection of an individual circulating sequence. However, any individual circulating sequence is likely to contain escape mutations that result in diminished T cell recognition relative to those generated against the initial infecting sequence(17) or a prototype sequence (106,107). Bole1a is designed to decrease the genetic distance between vaccine and circulating strains and to minimize the inclusion of escape mutations. Although the consensus sequence had shared identity for 13/15 epitopes, our head-to-head comparison of cons1a versus bole1a sequence at the two epitopes that differed demonstrated improved capacity to expand cross reactive HCV specific T cells responses using bole1a. One of the differences present in the consensus sequence represented a known escape mutation in an HLA A*01 restricted epitope. The HCV $NS3_{1436\text{-}1444}$ ATDALMGY

epitope has been shown to predominate in HLA A*01 populations, despite a known fitness cost (95).

Following vaccination against any virus, individuals subsequently exposed to that virus will have to respond to sequences similar but not identical to those they have already seen. Ability to expand T cell populations that recognize common circulating sequence variants will be critical if any individual sequence is used as a vaccine strain. Bole1a reliably expanded T cell responses against a broad array of circulating variants with greater magnitude of responses compared to naturally occurring sequence variants (Figure 4H). The bole 1a sequence can induce cross-reactive responses against many variants, including those not circulating in that host; an observation noted across subjects.

Although bole1a expanded T cells capable of recognizing many different circulating epitopes, some circulating variants were not recognized well by T cells expanded with any peptide sequence. For example, for the CINGVCWTV bole1a HCV $NS3_{1073\text{-}1081}$ peptide epitope, three of the four circulating variants of that sequence (CINGACWTV, CINGECWTI, and CINGECWTV) are very poorly recognized regardless of the peptide variant used to expand T cells(Figure 4F). Vaccination with any sequence is unlikely to induce a response to those sequences. Variant sequences have been shown to escape the T cell response through a number of mechanisms, including decreased binding to the MHC (44) or exploitation of a hole in the repertoire (52). How many T cell epitopes must be recognized to generate an effective T cell response is unknown. Thus, the impact of failure to generate a response to any one viral epitope is unclear. However, failure to generate a single T cell response is unlikely to render a vaccine ineffective.

Compared to T cells expanded with bole1a, T cell expansion with naturally occurring HCV variants generally resulted in diminished recognition of circulating sequence variants. While some variant sequences produced cross-reactive responses, others induced no cross-recognition. In addition, some variant sequences failed to induce any T cell response, making selection of a naturally circulating sequence problematic.

Bole1a contains a larger number of epitopes in the Immune epitopes database than did any circulating strain of HCV assessed (97). Experimentally, bole1a and 29 other sequences contain 10 of the 10 epitope sequences tested that were deemed most optimal for expansion and recognition, outperforming both cons1a and H77. The difference might have been greater had a larger number of epitopes been assessed. The H77 sequence contained nearly as many with 9 of the 10 optimal epitopes. This is not surprising given that H77 has been the most commonly used sequence for epitope discovery. Many of the tested epitopes were derived from this sequence in the generation of peptides to screen for T cell responses. Given the historical reliance on H77 for epitope identification, that bole1a contained even more optimal epitopes supports its use. In addition to the experimentally observed reliable expansion of cross-reactive, robust T cell responses, this theoretical assessment of immunogenicity also argues for the use of a representative sequence like bole1a.

The HIV literature debate over HIV strain selection has suggested use of multiple reference sequences, consensus sequences (85), most recent common ancestor sequence, or mosaic sequences. The STEP trial used *gag, pol, and nef* sequences from three reference strains in an Ad5-based vaccine and failed to reduce the incidence of infection, to lower HIV RNA set points (108), or to induce a difference in CD8+ T cell responses (109). Part of this failure has been attributed to the immunogenicity of the Ad5 vector in prime and boost, but selection of reference strains may also play a role. Vaccination of macaques has not found a consensus sequence to be particularly immunogenic compared to alternative methods (110). However, immune pressure on HIV is intense and consensus sequence contains escape mutations as a result. For example, the frequency of HIV variants in HLA B*57, HLA-B*27, and HLA-B*51 restricted sequences has been correlated with the prevalence of the restricting allele in populations, suggesting an evolving relationship between HIV sequence and HLA frequency (111). Finally, the use of computer generated sequences in vaccine design has been proposed for HCV (112) with coverage compared to reference strain H77, but direct testing of immunogenicity and cross-recognition had not been demonstrated previously. Our study provides

the first evidence that a computer generated HCV sequence can both expand cross reactive T cell responses and be recognized as antigen.

There are some limitations to our study. Lines generated against a sequence offer insight into recognition of sequence variants but may artificially expand one subset of the polyclonal population in a way that would not occur *in vivo*. In addition, we did not examine sequence cross-reactivity in multiple genotypes due to a relative infrequency of non-genotype 1 infections in our cohort. However, certain epitopes and escape variants are conserved across genotypes. For example, the escape variant Y1444F is the same variant in genotypes 1a, 1b, and 3a infection (95). Thus, our results may be relevant in non-genotype 1 infections. We are also limited to assessing responses that appear in the peripheral blood. Other studies have implicated an increased breadth and frequency in the CD8+ T cell response in the intrahepatic compartment versus the peripheral response (26,106). Thus, we may have underestimated the frequency of responses detected. Furthermore, we were unable to assess HLA-binding affinity for all known epitopes, including those of HLA-C as well as the B*27 and B*57 alleles. The presence of either the B*27 or the B*57 allele in HCV infected subjects is associated with increased frequency of HCV clearance (113).

We also demonstrate capacity to expand from previously HCV-exposed individuals rather than immunogenicity in HCV uninfected subjects. Ideally, vaccination of naïve hosts with every variant and artificial sequence would be performed. The assessment of the subsequent immune response would allow a more accurate comparison of immunogenicity. Given the lack of feasibility of such experiments, we assessed capacity to expand and cross-reactivity as a surrogate.

In conclusion, generation of a successful T cell vaccine will require a strain that is immunogenic as well as cross-reactive within genotype, or even better, with multiple genotypes. We have demonstrated for the first time the efficacy of a synthetic representative sequence, bole1a, to expand a robust and cross-reactive CD8+ T cell response and its superiority over other strategies. We anticipate that this strategy will help direct vaccine sequence selection for hepatitis C infection.

Table 1 HLA binding affinity and frequency of recognition of peptides.

Synthesized peptides encoding known HCV epitope sequences were tested in an *in vitro* radiolabeled competitive binding assay for binding to the MHC, and the 50% inhibitory concentration (IC50, nM) was determined. BBAASH subjects (n=60) with known HLA typing were screened at acute and chronic time points for responses against overlapping HCV peptides in an IFN-gamma ELISpot. Frequency of recognition of HLA restricted peptide epitopes by subjects with the matched HLA allele and subtype was determined. Subjects lacking the HLA allele required for presentation were excluded. Fraction recognition was calculated as follows: fraction recognition = (number of subjects with matched HLA allele recognizing epitope)/(total number of subjects with restricting HLA allele).

Epitope sequence	AA Position	MHC Allele	HLA binding (IC50)	# of HLA-matched subjects that recognize sequence	Number HLA-matched subjects	Fraction recognition
YLLPRRGPRL	35	A*02:01	125	0	33	0
GPRLGVRAT	41	B*07:02	15	5	15	0.33
RLGVRATRK	43	A*03:01	12	0	16	0
RLGVRATRK	43	A*11:01	6	0	5	0
RLGVRATRK	43	A*31:01	429	0	3	0
KTSERSQPR	51	A*03:01	69	0	16	0
KTSERSQPR	51	A*11:01	38	0	5	0
KTSERSQPR	51	A*31:01	67	0	3	0
DLMGYIPLV	132	A*02:01	80	3	33	0.09
LLALLSCLTV	178	A*02:01	357	1	33	0.03
FLVGQLFTF	285	A*02:01	67	1	33	0.03
RLWHYPCTI	614	A*02:01	23	7	33	0.21
RLWHYPCTV	614	A*02:01	12	11	33	0.33
RMYVGGVEHR	630	A*03:01	15	1	16	0.06
RMYVGGVEHR	630	A*11:01	8	0	5	0
RMYVGGVEHR	630	A*3101	95	0	3	0
LEDRDRSEL	654	B*40:01	426	1	3	0.33
LEDRDRSEL	654	B*40:02	4267	1	2	0.50
HPALVFDIT	881	B*07:02	160	1	15	0.07
RPALVFDIT	881	B*07:02	43	0	15	0
CINGVCWTV	1073	A*02:01	55	13	33	0.39
CTCGSSDLY	1123	A*0101	108	0	12	0
YLVTRHADV	1131	A*02:01	454	0	33	0
TLGFGAYMSK	1261	A*03:01	124	0	16	0
LGFGAYMSK	1262	A*03:01	136	0	16	0
LGFGAYMSK	1262	A*11:01	21	0	5	0
GIDPNIRTGV	1273	A*02:01	722	3	33	0.09

GVDPNIRTGV	1273	A*02:01	351	1	33	0.03
HPNIEEVAL	1359	B*35:01	99	1	5	0.20
HPNIEEVAL	1359	B*35:03	6.8	1	3	0.33
IPFYGKAI	1377	B*07:02	497	0	15	0
HSKKKCDEL	1395	B*08:01	764	7	13	0.54
KLVALGINAV	1406	A*02:01	5	14	33	0.42
ATDALMTGY	1436	A*01:01	1.3	5	12	0.42
SLMAFTAAV	1789	A*02:01	26	3	33	0.09
LLFNILGGWV	1807	A*02:01	4	0	33	0
ILAGYGAGV	1851	A*02:01	116	0	33	0
GVAGALVAFK	1858	A*03:01	26	0	16	0
GVAGALVAFK	1858	A*11:01	4	0	5	0
VAGALVAFK	1859	A*03:01	46	0	16	0
VAGALVAFK	1859	A*11:01	7	0	5	0
VLSDFKTWL	1992	A*02:01	305	6	33	0.18
DVVCCSMSY	2415	A*26:01	25	0	2	0
RLIVFPDLGV	2578	A*02:01	56	4	33	0.12
RVCEKMALY	2587	A*03:01	56	2	16	0.13
GLQDCTMLV	2727	A*02:01	18	3	33	0.09
SLLAPGAKQNV	4091	A*02:01	513	1	33	0.03

Table 2 Bole1a and consensus sequences.

Bole1a and cons1a sequences were compared for identity for known MHC class I restricted epitopes. The two epitopes for which bole1a (top) and the consensus (bottom) sequence differ are shown with the frequency of each amino acid at each position.

HLA	A.A. Location	Sequence								
		D	Y	P	Y	R	L	W	H	Y
		0.331	0.997	0.897	1	1	0.995	1	1	0.992
C*07E2	610-618	H								
		0.405								
		A	T	D	A	L	M	T	G	Y
		0.992	1	1	1	1	1	1	1	0.477
A*01NS3	1436-1444									F
										0.523

Table 3 Bole1a and Identified Variant Sequences

Subjects who recognized known epitope peptide sequences in an overlapping peptide ELISpot screen were followed longitudinally for sequence changes within the epitope region. Amino acid sequences for bole1a-encoded sequences with their positions in the HCV polypeptide and HLA restriction re written in full and are in bold with naturally occurring identified sequence mutations identified underneath by their changes away from the bole1a sequence.

HCV protein	A.A. Location	Peptide	HLA restriction
Core	1-9	**MSTNPKPQKK** ---D----R- --------R- --------RQ	A*11
Core	41-49	**GPRLGVRAT** --K------ --K---C-- -------T-	B*07
E1	285-293	**FLVGQLFTF** ------L-- ---S-----	A*02
E2	610-618	**DYPYRLWHY** H-------- H-D------ H-H------ H-S------ N--------	C*07
E2	614-622	**RLWHYPCTI** --------A --------T --------V	A*02
E2	621-628	**TINYTIFK** -------R	A*11
E2	654-662	**LEDRDRSEL** -----K-K-	B*60
p7	790-799	**LYGMWPLLLL** F---------	A*29/C*07
NS2	957-964	**RDWAHNGL** -N---S-L -S---DS- -S----S-	B*37
NS3	1073-1081	**CINGVCWTV**	A*02

		----A----	
		----E----	
		----E---I	
		--------I	
NS3	1111-1120	**WPAPQGARSL**	A*26
		-A----S---	
		------S---	
NS3	1359-1367	**HPNIEEVAL**	B*35
		-S-------	
NS3	1395-1403	**HSKKKCDEL**	B*08
		-------D-	
		----E----	
		--------F	
		---R-----	
NS3	1406-1415	**KLVALGINAV**	A*02
		--G-M-----	
		---G-V----	
		----M-----	
		--T-------	
		---T------	
		------V---	
		---V--V---	
NS3	1436-1444	**ATDALMTGY**	A*01
		--------F	

Table 4 HLA types of subjects in whom naturally occurring sequence variants were identified.

Cohort ID	HLA A1	HLA A2	HLA B1	HLA B2	HLA C1	HLA C2
17	*02:01	*03:01	*35:03	*51:01	*04:01	*14:02
18	*02:01	*02:01	*55:01	*44:03	*03:03	*04:01
24	*02:01	*03:01	*07:02	*57:01	*06:02	*07:02
26	*02:01	*11:01	*35:01	*52:01	*12:02	*15:02
28	*01:01	*02:01	*08:01	*37:01	*06:02	*07:01
29	*01:01	*02:01	*08:01	*13:02	*06:02	*07:01
30	*01:01	*02:01	*08:01	*08:01	*07:01	*07:02
45	*02:01	*02:02	*14:02	*15:16	*44:02	*05:01
46	*01:01	*03:01	*07:02	*08:01	*07:01	*07:02
47	*11:01	*32:01	*35:01	*40:02	*04:01	*15:02
49	*02:01	*30:04	*49:01	*51:01	*02:02	*07:01
50	*02:01	*24:02	*44:02	*51:01	*01:02	*05:01
51	*02:01	*02:01	*45:01	*51:01	*14:02	*16:01
52	*01:01	*01:01	*08:01	*08:01	*07:01	*07:01
53	*02:01	*30:01	*13:01	*37:01	*06:02	*06:02
57	*02:01	*02:01	*07:02	*15:01	*01:02	*07:02
109	*03:01	*03:01	*07:02	*08:01	*07:01	*07:02
111	*01:01	*26:01	*08:01	*51:01	*07:01	*15:02
113	*01:01	*02:01	*07:02	*08:01	*07:01	*07:02
115	[a]	[a]	[a]	[a]	[a]	[a]
116	*02:01	*24:02	*39:06	*57:01	*06:02	*07:02
133	*29:02	*31:01	*44:02	*51:01	*05:01	*14:02
139	*02:01	*11:01	*35:01	*52:01	*12:02	*15:02
145	*02:01	*02:01	*40:01	*44:05	*02:02	*03:04
148	*02:01	*02:01	*07:02	*44:03	*07:02	*16:01
154	*24:02	*31:01	*07:02	*51:01	*01:02	*07:02
160	[a]	[a]	*44:02	*44:03	*05:01	*16:01
171	*23:01	*33:03	*15:03	*53:01	*02:10	*04:01
175	*02:01	*03:01	*07:02	*15:01	*03:04	*07:02
177	*02:01	*30:02	*18:01	*40:01	*03:04	*05:01
181	[a]	[a]	[a]	[a]	[a]	[a]
269	*01:01	*02:01	*08:01	*08:01	*07:01	*07:01

[a] unavailable

Table 5 Epitope sequence data from subjects from whom T cell lines were derived.

Bold indicates visit at which T cell lines were used.

Subject	Duration of infection (days)	Epitope	Sequence
18	16	$E2_{614-622}$	RLWHYPCTI
	97		**n.d.**
	202		---------
	521		---------
	1158		--------V
	1499		--------V
18	16	$E2_{621-628}$	TINYTLFK
	97		**n.d.**
	202		--------
	521		--------
	1158		-V---I--
	1499		-V---I--
18	16	$NS3_{1073-1081}$	CINGVCWTV
	97		**n.d.**
	202		---------
	521		---------
	1158		---------
	1499		---------
18	16	$NS3_{1111-1120}$	WPAPQGARSL
	97		**n.d.**
	202		----------
	521		----------
	1158		------S---
	1499		------S---
18	16	$NS3_{1406-1415}$	KLVALGINAV
	97		**n.d.**
	202		----------
	521		----------
	1158		----------
	1499		----------
26	15	$NS3_{1406-1415}$	KLVALGINAV**
			------V---**
	104		**KLVALGINAV**
65	16	$NS3_{1436-1444}$	ATDTLMTGY
	379		----A---F
	702		----A---F
	1030		----A---F
	2374		**n.d.**
109	**539**	$Core_{41-49}$	*
	726		GPKLGVCAT

	993		--R---RT-
109	**539**	$E2_{610-618}$	*
	726		DYPYRLWHY
	993		---------
109	**539**	$E2_{621-628}$	*
	726		TINYTIFR
	993		--------
109	**539**	$p7_{790-799}$	*
	726		LYGMWPLLLL
	993		----------
109	**539**	$NS3_{1111-1120}$	*
	726		WAAPQGSRSL
	993		----------
109	**539**	$NS3_{1395-1403}$	*
	726		HSKKECDEL
	993		---R-----
113	18	$NS3_{1436-1444}$	ATDALMTGY
	57		--------F
	81		--------F
	114		**n.d.**
148	608	$E2_{610-618}$	**n.d.**
160	140	$NS3_{1073-1081}$	CINGACWTV
	251		**----V----****
			---------**
	308		---------
	462		----V----
	650		---------
	677		---------
	719		---------
181	**221**	$NS3_{1395-1403}$	**n.d.**

*aviremic

** both sequences present

n.d. = no data

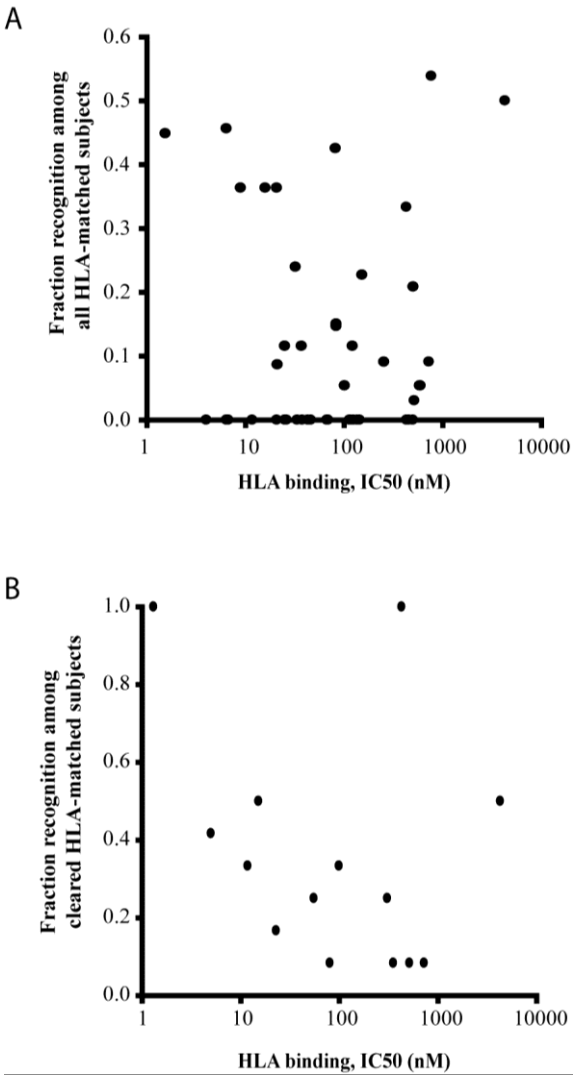


Figure 2 Lack of correlation between HLA binding affinity and frequency of recognition

The frequency of epitope recognition among 60 HCV infected subjects with matching HLA allele and subtype was assessed. HLA binding affinity was determined in an in vitro assay using purified HLA molecules with known allele and subtype. The percent of subjects recognizing HLA-matched epitopes as derived in Table 1 was graphed relative to the HLA binding affinity for (A) all subjects and (B) subjects that clear infection.

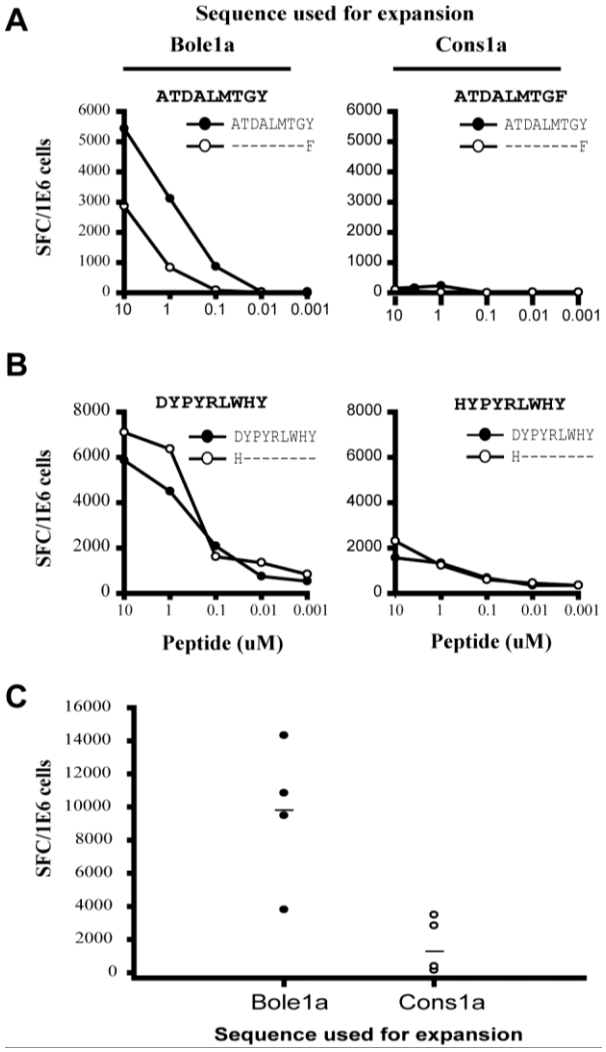


Figure 3 Relative to bole1a peptides, consensus peptides fail to or poorly expand T cells specific for peptides from either sequence.

PBMC were stimulated for twenty days with peptides bearing either the bole1a or consensus cons1a sequences. Stimulating peptide sequence is indicated on each graph in the upper right corner. The resulting lines were tested against titrated concentrations of peptides encoding the (--•--) bole1a or cons1a sequence (--o--) in duplicate wells. (A) HLA A*01 HCV NS3 1436-1444, subject 65 (B) HLA C*07 HCV E2 610-618, subject 109 (C) Summation of the total magnitude of response (Total SFC/1e6 cells).

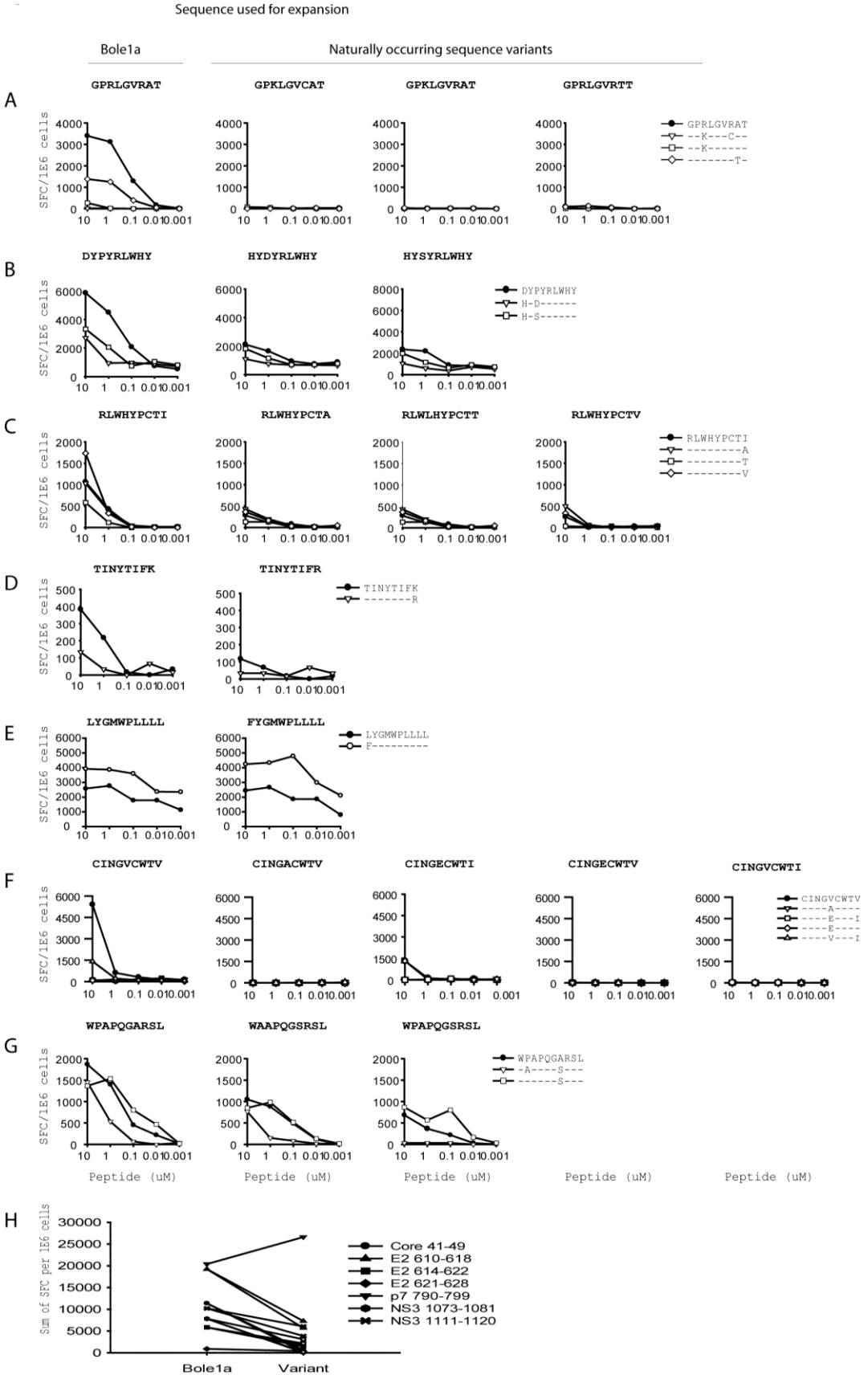

Sequence used for expansion
Bole1a
Naturally occurring sequence variants
A
GPRLGVRAT
GPKLGVCAT
GPKLGVRAT
GPRLGVRTT
GPRLGVRAT
--K---C--
--K------
-------T-
B
DYPYRLWHY
HYDYRLWHY
HYSYRLWHY
DYPYRLWHY
H-D------
H-S------
C
RLWHYPCTI
RLWHYPCTA
RLWLHYPCTT
RLWHYPCTV
RLWHYPCTI
--------A
--------T
--------V
D
TINYTIFK
TINYTIFR
TINYTIFK
-------R
E
LYGMWPLLLL
FYGMWPLLLL
LYGMWPLLLL
F---------
F
CINGVCWTV
CINGACWTV
CINGECWTI
CINGECWTV
CINGVCWTI
CINGVCWTV
----A----
----E---I
----E----
----V---I
G
WPAPQGARSL
WAAPQGSRSL
WPAPQGSRSL
WPAPQGARSL
-A----S---
------S---
SFC/1E6 cells
Peptide (uM)
H
Sum of SFC per 1E6 cells
Core 41-49
E2 610-618
E2 614-622
E2 621-628
p7 790-799
NS3 1073-1081
NS3 1111-1120
Bole1a
Variant

Figure 4 Relative to peptides encoding bole1a sequence, peptides encoding variant sequences fail to or poorly expand T cells specific for either bole1a or variant sequence. PBMC were stimulated for twenty days with a peptide of either the bole1a or an identified circulating sequence of that same epitope. In the upper right corner of each graph, the sequence used to expand is shown with amino acid differences from bole1a underlined. The resulting lines were tested against titrated concentrations of peptides in duplicate wells encoding the bole1a sequence (--•--) and all identified circulating variants of that epitope (open shapes) in an IFN-gamma ELISpot assay. (A) HLA B*07 HCV Core 41-49, Subject 109 (B) HLA C*07 HCV E2 610-618, Subject 109 (C) HLA A*02 HCV E2 614-622, Subject 18 (D) HLA A*11 HCV E2 621-628, Subject 109 (E) HLA A*29 HCV 97 790-799, Subject 109 (F) HLA A*02 HCV NS3 1073-1081, Subject 160 (G) HLA A*26 HCV NS3 1111-1120, Subject 109 (H) Sum of the magnitude of ELISpot response at peptide dilutions of 10, 1, and 0.1uM (y-axis) when peptides encoding bole1a or a naturally circulating variant was used to expand the T cells (x-axis). The lines connect the bole1a epitope to its corresponding variants.

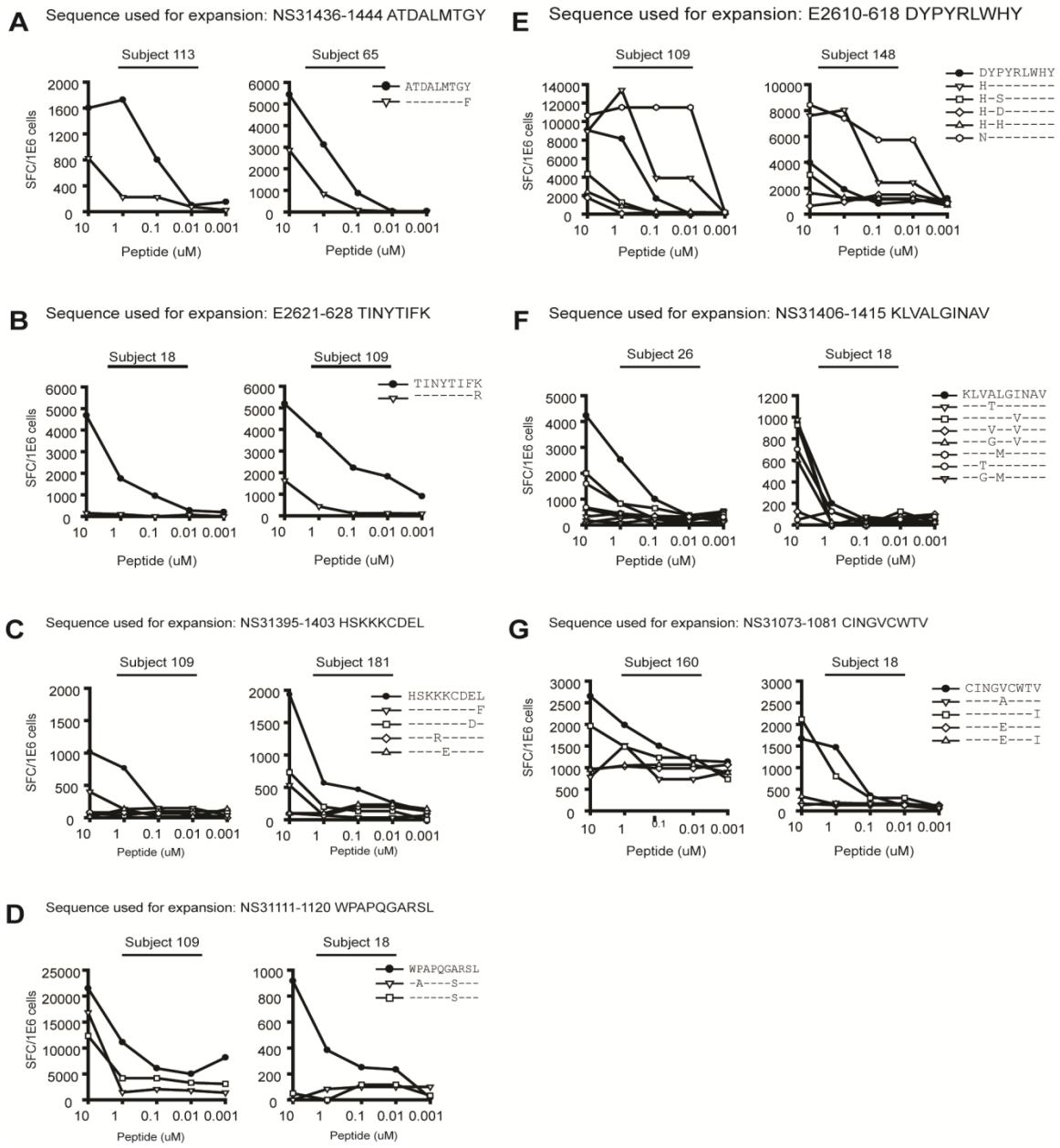


Figure 5 Bole1a reliably expands T cells with diverse cross-reactivity

PBMC were stimulated for twenty days with peptides bearing either the bole1a identified variant sequences. The resulting lines were tested against titrated concentrations of peptides encoding an HCV consensus sequence and identified variants in duplicate wells in an IFN-gamma ELISpot. (A) HLA A*01 HCV NS3 1436-1444, Subjects 113 and 65 (B) HLA A*11 HCV E2 621-628, Subjects 18 and 109 (C) HLA B*08 HCV NS3 1395-1403, Subjects 109 and 181 (D) HLA A*26 HCV NS3 1111-1120, Subjects 109 and 18 (E) HLA C*07 HCV E2 610-618, Subjects 109 and

148 (F) HLA A*02 HCV NS3 1406-1415, Subjects 26 and 18 (G) HLA A*02 HCV NS3 1073-1081, Subjects 160 and 18.

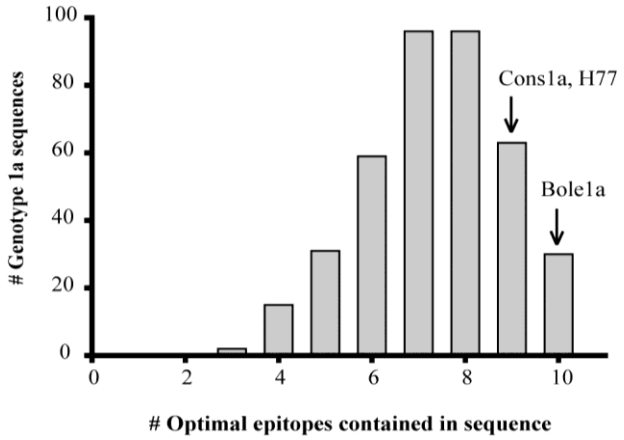


Figure 6 Bole1a sequence contains the greatest number of optimal epitopes

The optimal epitope sequence was defined as the sequence that induced the largest response or within 2-fold response of the largest response on IFN-gamma ELISpot at a peptide concentration of 1uM. Lines generated that produced only responses less than 100 SFC/1E6 cells were excluded from consideration. Full-length genotype 1a sequences culled from GenBank (n=390), cons1a, and bole1a sequences were then screened for the presence of the optimal epitopes and the total number of optimal epitopes per sequence was tallied.

Chapter 3

Introduction

Approximately 170 million people worldwide are infected with hepatitis C virus (HCV) worldwide (1), including 2.7 million in the United States (3). Upon HCV infection, approximately 2/3 of people will progress to chronic infection. Chronic HCV is the leading cause of hepatocellular carcinoma and liver transplantation (3,114) with acute infection often being asymptomatic. Direct-acting anti-viral medications not yet on the market promise sustained virological response rates of >90% without interferon use (73). However, such regimens are costly, and existing liver disease may continue to progress due to the existing severity of disease prior to therapy. As such, understanding the success and failure of the immune response may yield additional strategies to combat the virus in addition to enhancing vaccine responses.

The high error-prone rate of the HCV polymerase coupled with its rapid replication rate quickly generates a diverse quasispecies of genetically related viruses (13) with 10^{12} virions produced each day(115). Thus, a durable immune response must respond to and control a genetically flexible virus. Studies in chimpanzees and humans have linked spontaneous clearance with favorable genetic SNPs (116), production of cytokines (117), development of CD4 T cell responses (118), and a broad CD8+ T cell response (19,119). Conversely, failure of the CD8+ T cell response has been linked to a weak and narrowly focused response (37,72), mutations in viral sequence within known T cell epitopes (17,83), poor CD4 help (118,120), high numbers of Treg cells (121), and expression of co-inhibitory molecules including PD-1 (60,61,81), TIM-3 (122,123), LAG-3 (124), 2B4 (CD244) (124,125), KLRG1 (125,126), and CD160 (124,125). Studies in other chronic viral infections, including the lymphocytic choriomeningitis virus (LCMV) murine model (62,127) and HIV (82,128,129) have shown similar findings with an increasing number of inhibitory receptors correlating with diminished function (127). Whether additional inhibitory receptors function individually or synergistically to regulate adaptive immune cells during acute and chronic infection is not known, but blockade of one or more of the co-inhibitory molecules results in improved proliferation, expansion and cytokine function in some studies.

In general, it is thought that the severity of exhaustion correlates with the number of different receptors expressed, as well as the level of expression of each individual receptor (130). Functionally distinct subpopulations of exhausted $CD8^+$ T cells express unique combinations of inhibitory receptors that respond differently to inhibitory receptor blockade. In the LCMV model, chronic viral infection is associated with higher levels of viremia and greater number of inhibitory receptors that are sustained over time (127,130). However, in infections with frequent mutation, the role of antigen persistence in their coexpression has not been defined.

Inhibitory molecules such as PD-1 are up-regulated during acute HCV infection in individuals who clear as well as in those who progress to chronic infection (81). As such, it is possible that PD-1 is upregulated immediately following initial activation to ensure that the cell is refractory to further stimuli. Activation markers such as HLA-DR and CD38 have similarly been shown to be up-regulated in the setting of acute herpes virus infections, including human cytomegalovirus (CMV) and Epstein Barr Virus (EBV) (131,132). Similarly, CD38 expression increases during acute HCV infection with decreased expression over time (133).

Previous studies have examined the relationship of T cell dysfunction and circulating sequence mutations by comparing the identified circulating sequence to a reference sequence, such as a consensus sequence or a phylogenetic approach (97,134). Because acute HCV infection is often asymptomatic, most studies are unable to discern the infecting sequence prior to the onset of the adaptive immune response. Longitudinally following an acutely infected cohort (70) provides the unique opportunity to examine the concerted dance of viral sequence substitutions within T cell epitopes and T cell responses over time.

Given the role of inhibitory receptors in modulating T cell activation, it is also possible that inhibitory receptors shape the populations of effector and memory cells that develop during acute infections (135). Interleukin (IL)-7 signaling is essential to CD8 T-cell proliferation and function. The α chain of the IL-7 receptor (CD127) identifies effector CD8 T cells that will differentiate into memory cells with its selective expression identifying memory T cell precursors. During viral infection, CD127 expression on CD8 T cells occurs only when the pathogen is controlled so antigen is no longer present and sufficient CD4 T-cell help is available (136,137). Persistent viral

antigen is associated with low CD127 expression on primed T cells and correlates with exhaustion of a previously stable primed T-cell population (138). Therefore, we also assessed the relationship between CD127 expression and expression of inhibitory molecules.

We hypothesized that T cell activation and upregulation of inhibitory molecules requires on ongoing recognition of the virus with engagement of the T cell receptor. We conducted longitudinal viral sequencing of ten subjects with T cells available for flow cytometry staining for activation, co-inhibitory, and memory markers. Here, we present longitudinal data on CD8+ T cell activation, recognition of virus, and the relationship of both with memory and inhibitory receptor expression.

Methods

Subjects

The Baltimore Before and After Acute Study of Hepatitis (BBAASH) cohort of injection drug users (IDU) is a prospective study of injection drug users at risk for hepatitis C infection. Eligible participants have a history of or ongoing intravenous drug use and are seronegative for anti-HCV antibodies at enrollment. Written consent was obtained from each participant. Once enrolled, participants receive counseling to reduce intravenous drug use and its complications (70). Blood is drawn for isolation of serum, plasma, and peripheral blood mononuclear cells (PBMC) in a protocol designed for monthly follow-up. Participants with acute HCV infection were referred for evaluation of treatment. The study was approved by the Institutional Review Board at the Johns Hopkins School of Medicine.

For infected subjects in the BBAASH cohort, the date of initial viremia is defined as either 1) the first visit if HCV RNA is detectable in the absence of HCV Ab on enrollement or 2) the midpoint between the last RNA negative and first RNA positive date if initial viremia occurs on subsequent visits. For this study, we selected subjects from the BBAASH cohort who met the following criteria: 1) HCV Ab negative upon enrollment; 2) Initial viremia at the first visit (acutely infected on enrollment) or within 60 days of the last HCV RNA negative specimen in those with

viremia at later visits. ; 3) identified T cell responses by IFN-gamma ELISpot screen for which MHC multimers are available 4) T cells available for flow cytometry, 5) plasma available prior to the development of adaptive immune responses and 6) viremia at levels sufficient for HCV RNA sequencing at multiple time points.

Flow cytometry

PBMC were thawed in RPMI+50% FCS. Cells were stained for viability using the Cell Viability Dye (Invitrogen). Cells were stained for 20 min at room temperature with commercially available or custom-made APC-conjugated pentamers (ProImmune) with the exception of an APC-conjugated tetramer from the NIH Reagent Bank (HSKKKCDEL). Cells were additionally stained with the following antihuman fluorescent antibodies: anti-CD244 FITC, anti-TIM-3 PE (R&D), CD8 V450, PD-1 biotin (Mederex) and avidin-QDOT585 (Invitrogen), CD38 QDOT605 (Invitrogen), CD3 QDOT655 (Invitrogen), HLA-DR APC-H7.

Samples were run on a BD LSR-II instrument. Events for the entire content of the tube were collected with a minimum of 300,000 events per tube. Flow cytometry data was compensated in FACS Diva. Cells were gated on viable, CD3+ CD8+ cells in Flowjo. Gated data was exported to R where graphs and analysis was performed.

Hemigenome sequencing:

A 5.2 kb region spanning from Core through NS3 was amplified as described previously (98) with the exception that RT was performed using Superscript III (Invitrogen) and PCR was performed using Accuprime Pfx (Invitrogen). Amplicons were cloned into the pCR-XL TOPO vector using the TOPO-XL PCR cloning kit (Invitrogen). A 603 bp region from E1/E2 containing hypervariable region 1 was sequenced using the sequencing primer H77-1868a21 (5'-GAAGCAATAYACYGGRCCACA-3'). Sequence contigs were assembled in CodonCode Aligner (version 2.0.6, CodonCorp, Dedham, MA) and analyzed in BioEdit (version 7.0.9.0) with alignment using Clustal X. Phylogenetic trees were built based on these sequences, and a

representative clone nearest the center of the tree (139)was selected for further sequencing of the desired epitope.

Interferon-gamma ELISpot Assay

Ex vivo HCV CD8+ T cell responses were quantified by Human IFN gamma ELISPOT assays as previously described (37). Briefly, PBMC were screened for recognition of HCV-specific antigens using pools of overlapping peptides covering the entire HCV polyprotein and previously defined optimal epitopes. PVDF plates were coated with 2.5ug/ml recombinant human anti-IFN-gamma antibody (Endogen M-700A) in 100ul PBS/well at 37ºC at 4ºC overnight. Plates were washed with sterile PBS eight times before blocking with RPMI +10% FCS for 30 minutes. Either 20,000 or 30,000 cells in R10 media were added to the well. PHA served as a positive control. Plates were incubated for 20 hours at 37ºC with 5%CO2. Following incubation, plates were washed with 200ul sterile PBS eight times and blotted dry. Biotin-labeled anti-IFN-gamma (Endogen, M-701B, 0.25ug/ml, 100ul) was added to each well and incubated for 90 min at room temperature. Plates were washed and incubated with streptavidin-alkaline phosphatase (Bio-Rad 170-3554, 100ul) for 45 minutes at room temperature. Following additional washes, the plates were developed with BCIP/NBT Tris-buffer (pH 9.5) solution (Bio-Rad, 170-6532 and 170-6539) according to manual instructions. Plates were dried overnight and read on a Ziess ELISpot plate reader.

R analysis

Identification of longitudinal changes in epitope sequence were performed in R v 2.13.2 (140). Fasta files containing 5.2kb HCV sequences were aligned to the genotype 1a sequence, H77 in BioEdit and then converted to amino acid sequence. MHC Class I epitopes were identified based on their position in the H77 genotype 1a sequence. The sequence of these epitopes within the HCV sequences were extracted using the bio3d package (141) as well as unique code written in R. The initial infecting HCV sequence was defined as earliest representative sequence identified prior to 60 days of infection. This was chosen to select sequences that could be

identified prior to the onset of the adaptive immune response. Changes in sequence were identified as changes in amino acid sequence away from the initial infecting sequence. Where multiple clones were sequenced, both sequences were considered relative to the initial infecting sequence for potential mutations.

Line graphs and correlations were generated using the R base package. Data were organized using the reshape package(142). Boxplots and pie graphs were generated using the ggplot2 package (143).

Results

We identified 10 acutely HCV infected subjects who met all criteria including plasma available prior to the development of adaptive (T cells and antibody) immune responses, viremia at sequenceable levels, and subsequent development of multimer-quantifiable T cell responses. Subject characteristics are delineated in Table 6. We sequenced circulating HCV using a clonal sequence method and compared clones from later time points to the earliest sequenced virus for that individual (Table 7). We isolated and sequenced virus within the first 60 days of infection for all 10 subjects. Via flow cytometry, we assessed levels of activation molecules (CD38 and HLA-DR), a memory molecule (CD127), and inhibitory molecules (PD-1, TIM-3, and 2B4). These inhibitory molecules were selected based on their having detectable levels on peripheral T cells at some point in HCV infection. We then compared differences in surface expression of these molecules on HCV-specific T cells based on viral sequence variation and duration of infection. For seven subjects, we were able to isolate and stain T cells obtained during the acute phase of infection. However, for subjects 115 and 65, we were only able to isolate antigen-specific cells at 38 weeks and 339 weeks after initial infection, respectively.

When multiple clades of virus were found, as in chronic infection, multiple clones were sequenced. Given that any intact viral sequence could induce recognition and T cell receptor signaling, we considered sequence intact at any time points having any intact viral sequence even if that clone did not represent the majority of sequences.

Subjects in early acute infection show global activation of HLA-DR and CD38.

The earliest phase of acute HCV infection is characterized by viremia without T cell responses, which are first detected 6-8 weeks into infection (144). The total CD3+CD8+ T cell population as well as T cells specific for HCV (once detectable), CMV, and EBV antigens (pentamer positive) were phenotyped throughout the course of HCV infection. The results were correlated with antigen status: 1) no detectable viremia (absent), 2) viremic and specific antigen intact (intact) or 3) viremia but specific antigen mutated to an unrecognized form (mutated). Further subsetting of mutated antigens was done so that mutations that impair T cell recognition in vitro were separated from those that don't have effects on recognition that can be measured in an *in vitro* HLA binding affinity (145). Representative CD38 and HLA-DR staining is shown in Figure 7 for a robust T cell response to HCV. HCV specific T cell responses ranged in the first 180 days of infection from 0.01- 2.58% of total CD8+ T cells. We identified two subjects from whom we could isolate and stain cells in the hyper acute phase of infection. Surprisingly, 27-30% of the global CD8+ population dually expressed HLA-DR and CD38 at 6 and 7 weeks of HCV infection (Figure 8A and B), suggestive of bystander activation. These levels then diminished to 4-17% over time. This effect was true for antigen-specific cells specific for latent (EBV, CMV) or previously controlled (Influenza (Flu)) viruses as well. In these two subjects, 27-33% of EBV, CMV, and Flu-specific cells dually expressed HLA-DR and CD38 at weeks 6 and 7, percentages which declined to 0-15% at later time points.

In contrast to the earliest time points, the global CD8+ T cell population and T cells specific for control viruses generally did not show marked dual expression of HLA-DR and CD38 after the first 8 weeks of HCV infection (Figure 8C-J). There were some exceptions with dual expression seen on more than 25% of flu-specific cells from Subject 269 (weeks 11 and 49) and Subject 57 (weeks 13 and 33), CMV-specific cells from Subject 57 (week 47) and EBV-specific cells from Subject 18 (weeks 167, 244 and 314) and Subject 111 (week 12). In addition, Subject 115 expressed HLA-DR and CD38 on more than 50% of T cell specific for two CMV epitopes (IPSI and TPRV) at multiple time points despite having little expression of HLA-DR and CD38 on

EBV, influenza, and global CD8+ T cells (Figure 8H). None of the subjects was febrile or otherwise obviously ill at the time that the samples were collected.

Focusing on the HCV-specific response, HCV-specific CD8+ T cells showed high levels of activation and had higher level expression of activation markers than the global population with only a few exceptions. For Subject 175, 25% of T cells specific for the C63B HCV epitope had dual expression of HLA-DR and CD38 at week 6 with expression between that and 86% through week 99 (Figure 8A). CD8+ T cells from Subject 175 specific for the epitope E2 614-622 were not identified until week 29 but when identified, 84% dually expressed these activation markers, a number which declined steadily through week 99 of infection. HCV-specific cells identified also showed steady decline in dual expression of HLA-DR and CD38 from at week 6 in Subject 461 dually expressed HLA-DR and CD38 to week 33 (Figure 8B). Declining dual expression of HLA-DR and CD38 was observed on several other HCV-specific T cell populations identified outside the first 8 weeks of infection as well, although not uniformly (Figure 8C-J). We then asked if low levels of HLA-DR and CD38 on HCV-specific cells were due to mutations in the antigen that prevented T cell recognition.

HCV-specific CD8+ T cells show greater HLA-DR and CD38 expression when the circulating viral sequence is intact relative to the initially identified circulating sequence.

HLA-DR and CD38 have been identified as markers of activation. We hypothesized that coexpression of HLA-DR and CD38 would positively correlate with recognition of cognate antigen and would be negatively correlated with control of virus or substitutions within T cell epitopes that abrogate recognition. Although declining dual expression was observed on T cells specific for HCV in Subjects 175 and 461 despite persistent viremia with no mutations in targeted HCV antigens over time, the levels of activation remained high even in the late stages of infection (Table 7, Figure 8A, B). For Subject 26, 34% of cells expressed both HLA-DR and CD38 at week 15, when cognate antigen was intact, with a decrease to 20% of antigen-specific cells activated following clearance of viremia (Figure 8C). Due to low viremia at week 49 for Subject 269, viral sequencing could only be performed at weeks 11 and 20, when 29 and 38% of the HCV-specific

cells dually expressed HLA-DR and CD38, respectively (Table 7, Figure 8D). The HLA B*08-restricted epitope C171A mutation K1398R observed has been previously shown to permit ongoing T cell recognition, potentially permitting continued recognition and a high percent of C171A-specific T cells to be activated (107).

Subject 57 cleared initial infection and had an identified reinfection at week 47. T cells obtained during his initial infection as well as prior to and after his reinfection were phenotyped (Figure 8E). Unfortunately, due to the low level of viremia during his second infection, we were unable to sequence that virus. Interestingly, we found that HCV-specific cells for this individual showed persistently high level activation of T cells specific for two epitopes and low levels of activation of T cells specific for two other epitopes. Throughout the initial infection and reinfection, HCV-specific cells recognizing the 140G and the C63B showed consistently low coexpression levels with 7-15% expressing HLA-DR and CD38. The exception occurred at week 58, when 140G epitope-specific T cells had 29% dual expression. In contrast, T cells specific for the 4H and A2-61 epitopes showed higher levels of HLA-DR and CD38 dual expression, ranging from 15-50% of cells. Thus, activation patterns are not constant on T cells specific for all epitopes following control of infection and the activated state may persist for variable lengths of time following successful control of an infection.

High levels of activation, as evidenced by dual HLA-DR and CD38 expression, were not only seen following control of infection, but could also be seen late in chronic infection. In Subject 30, we identified substitutions at the 9^{th} position within the epitope for both HLA A*02 epitopes A2-61 and C63B (Table 7). For the epitope A2-61, a valine or isoleucine at amino acid position 622 binds the major histocompatibility complex with similar affinity. T cells lines generated against the E2 614-22 peptide RLWHYPCTI as well as the NS3 1073-1081 peptide CINGVCWTV retain the capacity to recognize the V_{622} and I_{1081} variant, respectively (146). Perhaps not surprisingly, 50 and 63% of T cells specific for these epitopes, respectively, maintained dual expression of HLA-DR and CD38 (Figure 8F). This suggests that ongoing recognition is possible, despite substitution within the HLA- binding region.

Retention of cognate antigen or reversion to the cognate sequence over time was not associated with a consistent pattern of activation. Subject 18 cleared an initial infection but became persistently infected following re-infection at week 24. T cells specific for three of four HCV epitopes showed increased dual expression of HLA-DR and CD38 at the time of reinfection, and two of these epitope retained high levels of activation through week 45 (Figure 8G). Notably, the sequenced virus in reinfection showed no changes in viral sequence from the initial viral sequence for the C63B or 140G epitopes (Table 7). However, the effects of ongoing stimulation differed in that T cells specific for C63B maintained high level activation, but T cells specific for 140G did not. In Subject 115, who became chronically infected with the first HCV infection, 80% of CD8+ T cells specific for the 140G epitope showed high levels of HLA-DR and CD38 expression at week 38, which declined to 19% by week 57 and 0% by week 93 (Figure 8H). A circulating substitution within the 140G epitope was identified at the K1406N position at week 64 with reversion to the initial infecting sequence at week 93, suggesting that other factors in addition to viral sequence variation may play a role.

Both Subjects 115 and 65 were found to carry the Y1444F mutation relative to the initial infecting sequence in the 143D epitope (Table 7). The predominant Y1444F substitution impairs binding to the HLA-A*01 molecule, but carries a fitness cost so reversion to Y is common is the absence of T cell pressure (95). In both cases, HLA-DR and CD38 expression on CD8+ T cells specific for the 143D epitope ranged from 5-21% (Figures 8 H and I), which suggests that the F substitution is maintained by ongoing activated CD8 T cell pressure. While Subject 111 also targets the 143D epitope, there is no significant dual expression on T cells specific for that epitope despite the presence of F in the last position. However, that subject was infected with 143D bearing F in the last position and may not ever have activated T cells specific for the better recognized Y variant.

Figure 9 shows HLA-DR and CD38 dual expression frequency for all subjects combined according to the virus targeted versus the general CD8+ T cell population. Collectively, the global CD8+ T cell population and T cells specific for EBV and resolved influenza viral infection showed low levels of HLA-DR and CD38 (Figure 9A). CMV-specific CD8+ T cells showed a greater range

of HLA-DR and CD38 dual expression. (Figure 9A) The percent of HLA-DR and CD38 coexpression was more variable on HCV specific T cells. Overall, we detected higher levels of HLA-DR and CD38 coexpression when the viral sequence was intact than when the viral sequence contained substitutions or when the virus was controlled (undetectable HCV RNA). Mutations known to impair T cell recognition *in vitro* were associated with less dual expression. Interestingly, differences in HLA-DR and CD38 coexpression were even greater when stratified by prior to or after 180 days of infection (Figure 9B), suggestive of an initial activation in the setting of acute infection that decreases over time. Given that increased duration of infection has been hypothesized to result in upregulation of progressively more inhibitory receptors that decrease T cell proliferation and cytokine production, we assessed expression of such inhibitors.

Subjects in early acute infection showed elevated global levels of co-inhibitory markers.

Co-inhibitory markers such as PD-1, TIM-3, and 2B4 have been shown to be expressed at high levels on T cells in subjects who progress to chronic infection. We hypothesized that inhibitory receptor expression is coordinately upregulated and maintained over time and correlates inversely with high levels of activation. We examined PD-1, TIM-3, and CD244 (2B4) expression on the global CD8+ population, CD8+ cells specific for the latent viruses CMV and EBV, CD8+ cells specific for prior influenza infection, and HCV-specific cells. Representative flow cytometry is shown in Figure 10A with T cells from subjects stained longitudinally in Figures 11A-J.

The earliest phase of the adaptive immune response demonstrated minimal increase in the number of co-inhibitory receptors expressed on the global CD8+ T cell population versus later time points in HCV infection. In the two subjects with cells isolated before 8 weeks of infection, approximately 11% of the global CD8+ T cell population expressed two or three co-inhibitory receptors at this time point, a number which declined to less than 5% expressing two receptors and no expression of three inhibitory receptors in the subsequent weeks (Figure 11A and B). Interestingly, T cells specific for EBV, CMV, and influenza also had maximal co-inhibitory receptor expression during this earliest acute phase of HCV infection. At less than 8 weeks of infection,

24% of EBV-specific cells, 26% of CMV-specific cells, and 50% of influenza-specific cells expressed either two or three co-inhibitory molecules. By 29-33 weeks of infection, the percent of control-virus specific T cells with at least two co-inhibitory receptors declined to 14% for flu (Figure 11A) and 10% or 4% for EBV and CMV, respectively (Figure 11B).

Notably, in Subject 175, 75% of cells specific for the HCV epitope C63B had no expression of any inhibitory receptors at 6 weeks of infection compared to 37% of cells specific for that same epitope at week 7 for subject 461. Subject 175 demonstrated an increase in the number of co-inhibitory receptors between weeks 6 and 53, before declining by week 99 (Figure 11A). For Subject 461, however, the percentage of HCV-specific cells that expressed either two or three co-inhibitory receptors peaked at week 7 with 35 and 61% for the epitopes 4H and C63B before declining to 5 and 22%, respectively, by week 33 (Figure 11B). Both subjects became persistently infected so the difference in coinhibitory receptor expression was not associated with a different infection outcome.

Expression patterns of inhibitory T cell receptors change variably over time, are not progressively (or sequentially) upregulated and may correlate with antigen-status

We found that the patterns of co-inhibitory molecules on HCV-specific cells varied by subject as well as by epitope but generally declined over time, regardless of outcome. Such patterns did not clearly correlate with the presence or absence of viremia in those who cleared infection. Subjects 18, 26, and 57 cleared their initial viremia (Table 6). In the case of Subject 18, the number of co-inhibitory receptors remained constant on T cells specific for the 127D, 140G, and A2-61 epitopes (Figure 11H). In contrast, the percentage of T cells specific for C63B with at least two co-inhibitory receptors declined from 27% in week 14 to 8% in week 111, an aviremic time point between his first and second infection. However, this percentage increased by weeks 244 and 314, over 60 and 110 weeks into his second infection. The sequence of virus in epitope C63B remained intact at all points tested, so loss of antigen neither explained the difference between T cell inhibitory receptor expression levels over time nor between epitopes in the same subject. Subject 26 had 19% of HCV-specific T cells with two or three co-inhibitory receptors

during infection with a decline to 11% following clearance of infection, but that decline was driven by loss of cells with two receptors since the percent of cells with three receptors increased slightly (Figure 11C). For Subject 57, T cells specific for epitopes 4H and A2-61 showed greater expression of two or more inhibitory receptors at 33 and 47 weeks than during initial infection, but that was not true of T cells directed against two other HCV epitopes recognized by the same subject. The dual expression of two or three inhibitors declined through week 68 but increased again at week 166, despite the absence of detectable viremia after week 47 (Figure 11E).

Not only did patterns of inhibitory receptor expression not differ between those who controlled infection and those who did not, there was neither a consistent increase in the number of inhibitory receptors expressed over time with chronic HCV infection nor a correlation with maintenance of intact antigen and higher numbers of coinhibitory receptors being expressed throughout infection. Subject 30 had identified substitutions at the 9th position within the two HLA A*02 epitopes studied. For the A2-61 epitope, at weeks 95 and 192, only 3-7% of HCV-specific T cells had more than one inhibitory receptors present. However, at weeks 261 and 425, these numbers increased to a range of 10-13%. In comparison, T cells recognizing the C63B epitope had 36% of cells with at least two co-inhibitory receptors, a number which declined to 6% by week 425 (Figure 11F). It is possible that the *in vivo* effects of these mutations differed between the two epitopes, explaining their different pattern over time. However, the effect of mutation on coinhibitory molecule expression does not appear to be consistent across epitopes, even in the same subject.

This point is further illustrated by Subject 115, where the 140G epitope undergoes mutation between week 38 and 64 with reversion to the initial infecting sequence by week 93. In the same subject, the 143D mutates from Y to F in the last position between weeks 3 and 38 of infection and remains stable throughout the infection (Table 7). T cells specific against the 140G showed declining levels of double and triple expression (around 48% initially) while T cells specific for the 143D epitope, initially expressed at least two co-inhibitory molecules on only 14% of cells which declined to 4% by week 93 (Figure 11H). Similarly, only 5% of T cells specific for the 143D epitope from Subject 65, who also maintains the Y1444F mutation (Table 7) have two

or more co-inhibitory receptors (Figure 11 I). Although the circulating virus has phenylalanine in the 1444 position for Subject 111 (Table 7), this was the sequence initially isolated at week 6.Only 1-3% of T cells express more than one co-inhibitory receptor. In comparison, 40% T cells specific for the C171A epitope express more than one co-inhibitory receptor at 12 weeks, a percentage that decreased to 0% at 29 weeks (Figure 11J). For subject 269, 20% of HCV cells specific for the C171A epitope expressed two inhibitory receptors at 11 weeks, a number which declined to 15% at week 49, but increasing the number of cells expressing three co-inhibitory receptors from 0 to 5% (Figure 11D). Altogether, these snapshots suggest a far more complex picture with some decline over time of inhibitory molecule expression rather than progressive increases in the number of coinhibitory receptors as has been shown in murine LCMV (130).

Analysis of inhibitory receptor expression by antigen status reveals more complex regulation.

We then grouped the circulating viral sequence by epitopes status: intact, mutated, low HCV RNA (detectable but not sequenceable), or absent (undetectable HCV RNA) relative to the initially identified circulating virus and CD8+ T cells based on expression of inhibitory receptors. Antigen status is shown with the percent single expression of PD-1, TIM-3, or 2B4 in Figure 12A, expression of two of the three—either PD-1/TIM-3, PD-1/2B4, or TIM-3/2B4 in Figure 12B, or triple expression of all three markers in Figure 12C. Overall, antigen-specific and global CD8+ T cells had low single expression of PD-1 with the most single-expression being found on HCV-specific cells where the circulating viral sequence was absent relative to the initially identified infecting sequence (Figure 12A). Interestingly, influenza-specific cells stained highly for TIM-3 single expression whereas CMV- and EBV-specific cells expressed higher levels of 2B4. In HCV infection, high frequency coexpression of PD-1 and TIM-3 (>12% of antigen specific T cells) occurred exclusively when the virus was intact (Figure 12B, first panel) One flu exposed subject, also had more than 12% of T cells coexpressing PD-1 and TIM-3, but high frequency PD-1/TIM-3 coexpression was not observed on EBV-specific, CMV-specific, or the general CD8 T cell populations of the same subject. PD-1/2B4 coexpression was detected at higher levels on EBV-specific, CMV-specific, and HCV specific T cells than on the general CD8 T cell population with

lower percent coexpression on HCV-specific T cells when the antigen was intact.(Figure 12B, middle panel) This suggests that continued antigenic stimulation is not associated with upregulation of both inhibitory molecules. Coexpression of TIM-3 and 2B4 was infrequent, with only five T cell populations, three of which were CMV-specific T cells, having >15% of cells with dual TIM-3 and 2B4 expression (Figure 12B, last panel). No subject had HCV-specific T cells with more than 15% TIM-3 and 2B4 coexpression.

Across all subjects and time points, less than 7% of the global CD8+ population expressed two or all three co-inhibitory receptors outside of the initial 6-7 weeks of infection (Figures 12B and 12C). Some subjects with intact antigen have a small percent of antigen specific T cells with triple expression of PD-1/TIM3/2B4 (Figure 12C). Thus, cognate antigen may be associated with the highest levels of expression, but is not sufficient to induce expression of all three inhibitory receptors.

Expression patterns of inhibitory T cell receptors correlate with dual expression of HLA-DR and CD38.

No definitive pattern clearly emerged as a signature of CD8+ HCV-specific T cells after dividing based on the absence of viremia, low VL, or intact antigen, or presence of substitutions. Given the increasing complexity of the quasispecies in chronic infection, we hypothesized that the dual combination of HLA-DR and CD38 may better illuminate the role of the co-inhibitory receptors and CD127. Preliminary analysis of HLA-DR and CD38 separately showed weaker trends (data not shown). Representative T cell staining for CD127 is shown in Figure 13. We compared the percentage of cells dually expressing HLA-DR and CD38+ and the expression of 2B4, PD-1, TIM-3, and CD127 and combinations of PD-1, TIM-3, and 2B4 (Figure 14). Due to the generally low expression of CD127 on HCV-specific cells, we did not examine the relationship between CD127 and co-inhibitory receptor expression. HLA-DR/CD38+ dual expression weakly correlated with PD-1 expression (Figure 14A, r^2=0.145, p=8.01e-5), TIM-3 expression (Figure 14B, r^2=0.229, p=3.54e-7), and 2B4 expression (Figure 14C, r^2=0.169, p=1.81e-5). HLA-DR and CD38 expression was strongly inversely correlated with CD127 expression (Figure 14D, r^2=0.248,

p=9.88e-8). Interestingly, a strong correlation was seen between HLA-DR/CD38 dual expression and dual PD-1/TIM-3 expression (Figure 14E, r^2=0.367, p=1.5e-11), with weak or no correlation seen between HLA-DR/CD38 dual expression and dual PD-1/2B4 (Figure 14F, r^2=0.0974, p=0.0014), or dual TIM-3/2B4 (Figure 14G, r^2=0.0206, p=0.15) expression. However, the strongest positive correlation was seen between HLA-DR/CD38 and triple expression of PD-1, TIM-3, and 2B4 (Figure 14H, r^2=0.367, p=1.5e-11). This suggests a relationship between activation and expression of co-inhibitory receptors, a surprising finding suggestive of activation not exhaustion when all three are expressed. We then looked at activation state (as measured by IFN-γ production) in association with recognition over time.

Functional responses do no track along CD8+ T cell responses.

In the setting of HCV infection, the breadth and frequency of the CD8+ T cell response has been shown to decline over time (37,147). HCV-specific T cell responses are generally of lower magnitude than are T cell responses to HIV (148). T cell responses to HCV are particularly infrequent and of low magnitude in the setting of chronic infection. The low precursor frequency of these antigen-specific cells precluded us from performing intracellular cytokine staining. At each visit where pentamer staining was performed, we screened our subjects for functional responses by IFN-gamma ELISpot. Despite maintaining clearly identifiable populations of antigen-specific T cells by pentamer staining, most subjects lost their functional responses in chronic infection (Figure 15). Thus, irrespective of expression of the three molecules associated with exhaustion that we assessed, there is loss of gamma interferon production after 100 weeks of infection of most HCV-specific CD8 T cell responses. The exception after 100 weeks of infection included the response to the C171A epitope in Subject 269. T cells specific for that epitope did express all three inhibitory co-receptors at time points where IFN-γ was produced in response to antigen so the maintenance of gamma interferon expression was not related to failure to upregulate these molecules. T cell gamma interferon production was more durable in subjects who controlled infection, suggesting active suppression of T cell function in persistent infection.

Discussion

We hypothesized that T cell dysfunction requires ongoing recognition of the virus to maintain an activated state and up regulation of co-inhibitory receptor expression. We performed longitudinal sequencing and flow cytometry on subjects to profile the relationship between viral sequence and cell surface phenotype. Much of our work hinges upon careful dating of the initial infection. Therefore, we selected subjects with frequent follow up to determine an estimated date of infection.

We had two subjects with available samples at 6-7 weeks. Surprisingly, a large percentage of the global CD8+ population as well as T cells specific for controlled latent viruses, and cleared influenza virus showed high levels of co-expression of HLA-DR and CD38, suggestive of bystander activation that diminished over time. Notably, this was not present after week 11 of infection. In humans, bystander activation and attrition have been suggested in the context of acute primary EBV infection in the first 30 days of infection with elevated HLA-DR, CD38, and granzyme B expression on CMV and influenza-specific cells (132). Similarly, bystander activation with elevated CD38 expression has been proposed in the context of primary HIV infection in the setting of detectable RNA but prior to seroconversion (149). CMV-specific cells have further been characterized to have elevated PD-1 and low production of IFN-γ in the setting of acute HBV infection (150). This may be attributable to the fact that T cells specific for those infections are frequent. However, HCV-specific T cells are of low frequency in virtually all individuals and account for less than 5% of the total CD8 T cell population in virtually all cases.

Although high CD38 expression has been previously described during the acute phase of HCV infection(133), we identified 5 out of 7 subjects with samples obtained after more a year of infection that still expressed HLA-DR and CD38 on HCV-specific T cells (Figure 10 A, E, F, G, and H). This suggests that ongoing activation of HCV-specific T cells is possible late in infection despite failure to produce IFN-γ *in vitro* upon stimulation. We had hypothesized that viral sequence and ongoing recognition may be the driving force. Consistent with that hypothesis, we saw higher co-expression of HLA-DR and CD38 on HCV-specific T cells where the cognate antigen was intact. We observed higher levels in the first 180 days of infection, but these high

levels were maintained relative to the global CD8+ population after 180 days of infection. In a prior study examining CD38 on HCV-specific cells, CD38 was present on HCV specific T cells in acute infection but was not present in different subjects analyzed during chronic infection. (151). However, HCV sequence was not determined for these subjects. Given our data that CD38 and HLA-DR are not maintained on T cells specific for mutated antigen, it is probable that these subjects developed substitutions within the T cell epitope that precluded recognition. Mutations that don't impair recognition by T cells in vitro were associated with T cell activation. What drives maintenance of these mutations *in vivo* is unclear since T cells seem to continue to recognize them and these viral variants persist in infected hosts. Intriguingly, recent vaccination studies in chimpanzees demonstrated that expression of HLA-DR with or without CD38 was correlated with improved proliferation and likelihood of clearance of HCV following vaccination (152). Whether this is just a marker of activation or of improved immune response remains to be seen.

Our data showed an inverse relationship between HLA-DR and CD38 co-expression and CD127 expression (Figure 14D). This is consistent with previous work in HCV in which CD127 (IL-7Ralpha) are also down-regulated in chronically infected individuals and particularly on T cells whose cognate antigen has not undergone substitutions (153). The presence of HCV-specific CD127+ cells predicted clearance in chimpanzees (154) and humans (155). However, because IL-7 is critical for the maintenance of memory T cell populations, it is unclear whether the presence of CD127 is causative or reflective of an already durable immune response.

We did not see a clear signature of co-inhibitory molecule expression on the cell surface of antigen-specific T cells. There is a substantial body of literature on cross sectional analysis of individually inhibitory molecule expression in HCV infection with less on coexpression of multiple co-inhibitory molecules or longitudinal analysis. Our studies are the first to take into consideration the sequence of the virus to determine if there is continued production of cognate antigen. PD-1 has been shown to be more likely to be expressed on HCV-specific T cells in the setting of acute infection (61,81), and up regulation of multiple inhibitory molecules has been associated with progression to chronic infection(122). Chronically infected patients have been shown to up regulate PD-1, 2B4, KLRG1, and CD160 (125) on HCV-specific CD8+ cells in the periphery.

Moreover, the patterns of co-inhibitory molecules differs based on organ site with higher levels of PD-1 and 2B4 found in the liver compared to the peripheral blood in chronically infected livers (124).

The initial sequence identified in circulation among our subjects largely corresponded with the previously established bole1a sequence. The bole 1a sequence is a synthetic, representative clone that is phylogenetically derived using a primarily Bayesian approach (97). Such a sequence has been thought to minimize the degree of genetic dissimilarity between subjects. Differences from the bole1a sequence seen at initial infection arose primarily within the E2 614-622 (A2-61) epitope at the 9th position within the epitope (I622A/V), the 143D epitope (Y1444F) and in the C171A epitope (K1398R, Table 7). The high level of activation of T cells primed with sequence that matches bole1a is consistent with it being a potent antigen (146)

We demonstrate that HLA-DR and CD38 expression is associated with PD-1, TIM-3, dual expression, as well as triple expression in conjunction with 2B4. This suggests that ongoing activation is maintained despite the expression of the co-inhibitory receptors. Given that the inhibitory receptors are supposed to decrease activation of T cells, this is a surprising finding. Our data also demonstrate ongoing expression of CD38 and HLA-DR on HCV-specific T cells, suggesting that even late into chronic infection, populations of HCV-specific T cells continue to be activated in response to antigen despite the lack of detectable gamma-interferon production in late phases of infection. In total, this may suggest that expression of CD38 and HLA-DR may indicate recent activation but not the capacity of the T cell to produce cytokines.

T cell breadth and frequency are known to decline following the acute phase of infection (156,157) and one hypothesis offered has been the progressive upregulation of inhibitory molecules. In the LCMV murine model, it has been postulated that there is a temporal relationship between time of infection and expression of co-inhibitory receptors. In an elegant comparison of three strains of LCMV infection, infection with Clone 13 resulted in the greatest number of co-inhibitory receptors, compared with the acute infectious Armstrong strain or the intermediate T strain (127). Arguing against this hypothesis, we were able to identify T cells in acute infection with up regulation of multiple co-inhibitory molecules and T cells in late infection without high

percents of expression of two or more inhibitory molecules. In fact, many subjects had a gradual decline in the number of co-inhibitory receptors expressed. Perhaps not surprisingly given the complexity of the HCV quasispecies, we did not find a clear pattern of either up regulation or down regulation of co-inhibitory receptors on our subjects. Because we did not stain for classic memory markers, we don't know if the cells being analyzed are memory or effector cells. In addition, it is unclear whether these cells late into chronic infection represent the same population of T cells initially activated and expressing co-inhibitory receptors or whether this population reflects priming of a different T cell population later in infection.

Limitations of our study include that we were only able to profile ten subjects for longitudinal sequencing and comprehensive flow cytometry, of which only seven had available samples in the early acute (less than 8 weeks) phase of infection. Given that small sample size and multiple time points for each subject with multiple molecules assessed, statistics were complicated by the number of variables versus compared to the number of subjects. Furthermore, while we had clearly detectable antigen-specific T cells by pentamer staining, we were unable to perform intracellular cytokine staining to directly assess function along with cell surface phenotype and had to use ELISpot detection of IFN-γ as a surrogate.

We categorized subjects based on whether there was circulating antigen and whether this antigen sequence was intact. If any intact antigen were present in any number of clones, we labeled that visit as having intact antigen. We reasoned that any T cells exposed to circulating virus could have the potential to be activated if a small fraction of the circulating sequence maintained intact cognate antigen. However, it is unclear whether a certain threshold of a quasispecies must have the same viral sequence to impact T cell function so this may not have been the correct way to code a specimen. Further complicating the analysis, although mutation more commonly arise in the setting of T cell pressure (17,107,158), their impact on T cell recognition is variable. The 143D epitope (HLA A*01 NS3 1436-1444) reduces viral fitness by reducing helicase function 10-fold (95). However, this substitution is extremely common in the HLA A*01 population, as the mutant Y1444F sequence reduces binding to the HLA by an estimated 1000-fold (146). In contrast, substitutions in A2-61 epitope (HLA A*02 E2 614-622) at

I622V occur in a known HLA binding motif but does not impair expansion or recognition in ex vivo-expanded T cell lines (146). Thus, the effect of substitutions on T cells is highly variable and likely relates balancing constraints on viral fitness. Therefore, categorizing circulating sequences as containing substitutions or intact antigen underestimates the complexity of the impact of the substitutions. We did analyze effects of inhibitory molecules in the presence of mutations taking into account known mutations where possible. An additional complication is the variable data on 2B4 as a costimulatory molecule rather than a coinhibitory molecule on effector T cells (135,159). Thus, the effects of expression of 2B4 may be context dependant, further complicating analysis of the effects of its expression.

Overall, we determined that dual expression of HLA-DR and CD38 occurs in the setting of ongoing T cell recognition. Expression of both of these markers is associated with expression of greater expression of co-inhibitory receptors singly or in combination. Our work suggests that the role of the co-inhibitory receptors in making T cells less able to release the antiviral cytokine gamma interferon release is a complex process in which ongoing T cell recognition and activation is required, but not sufficient.

Table 6 Characteristics of subjects selected for longitudinal sequence analysis and flow cytometry

All subjects were uninfected with HIV or HBV.

ID	Genotype of first infection	Race	Gender	Outcome
18	1a	W	M	Clearance - reinfected
26	1a	W	M	Clearance
30	1a	W	F	Chronic
57	1a	W	M	Clearance - reinfected
65	1a	W	M	Chronic
111	1a	W	F	Chronic
115	1a	W	M	Chronic
175	1a	W	M	Chronic
269	1a	W	F	Chronic
461	1a	W	M	Chronic

Table 7 Sequence data for subjects

A 5.2kb region was amplified using a nested PCR technique and cloned into pCR-XL TOPO vector. A region of Core through E1 was sequenced to select a phylogenetically representative clone prior to sequencing of the region containing the epitope of interest. Where multiple clades were present, multiple clones were sequenced.

ID	Pentamer	HLA	Protein	AA Position	Sequence	Length Viremia (weeks)
18	A2-61	A02	E2	614-22	RLWHYPCTI	2
					--------V	214
					---------	244
					---------	314
	C63B	A02	NS3	1073-81	CINGVCWTV	2
					---------	214
					---------	244
	127D	A02	NS3	1273-82	GIDPNIRTGV	2
					-A--------	214
					-A--------	244
					-A--------	314
	140G	A02	NS3	1406-15	KLVALGINAV	2
					----------	214
					----------	244
					----------	314
26	140G	A02	NS3	1406-15	KLVALGINAV	2
					----------	15
30	A2-61	A02	E2	614-22	RLWHYPCTV	2
					--------I	95
					--------I	192
					--------I	261
					--------I	425
	C63B	A02	NS3	1073-81	CINGVCWTV	2
					--------I	95
					--------I	192
					--------I	261
					---------	425
57	4H	B07	Core	41-49	GPRLGVRAT	6
	A2-61	A02	E2	614-22	RLWHYPCTA	6
	C63B	A02	NS3	1073-81	CINGVCWTV	6
	140G	A02	NS3	1406-15	KLVALGINAV	6

65	143D	A01	NS3	1436-44	ATDTLMTGY	2
					--------F	339
					--------F	375
					--------F	418
111	C171A	B08	NS3	1395-1403	HSKRKCDEL	6
					---K----F	12
					--------V	29
	143D	A01	NS3	1436-44	ATDALMTGF	6
					V--------	12
					---------	12
					---------	21
					---------	29
					---------	58
115	140G	A02	NS3	1406-15	KLVALGINAV	3
					----------	38
					N---------	64
					----------	93
	143D	A01	NS3	1436-44	ATDALMTGY	3
					--------F	38
					--------F	64
					--------F	93
175	A2-61	A02	E2	614-22	RLWHYPCTA	0
					---------	53
					---------	69
					---------	99
	C63B	A02	NS3	1073-81	CINGVCWTV	0
					---------	6
					---------	53
					---------	69
					---------	99
269	C171A	B08	NS3	1395-1403	HSKKKCDEL	3
					---R-----	49
461	4H	B07	Core	41-49	GPRLGVRAT	0
					---------	7
					---------	17
	C63B	A02	NS3	1073-81	CINGVCWTV	0
					---------	7
					---------	17

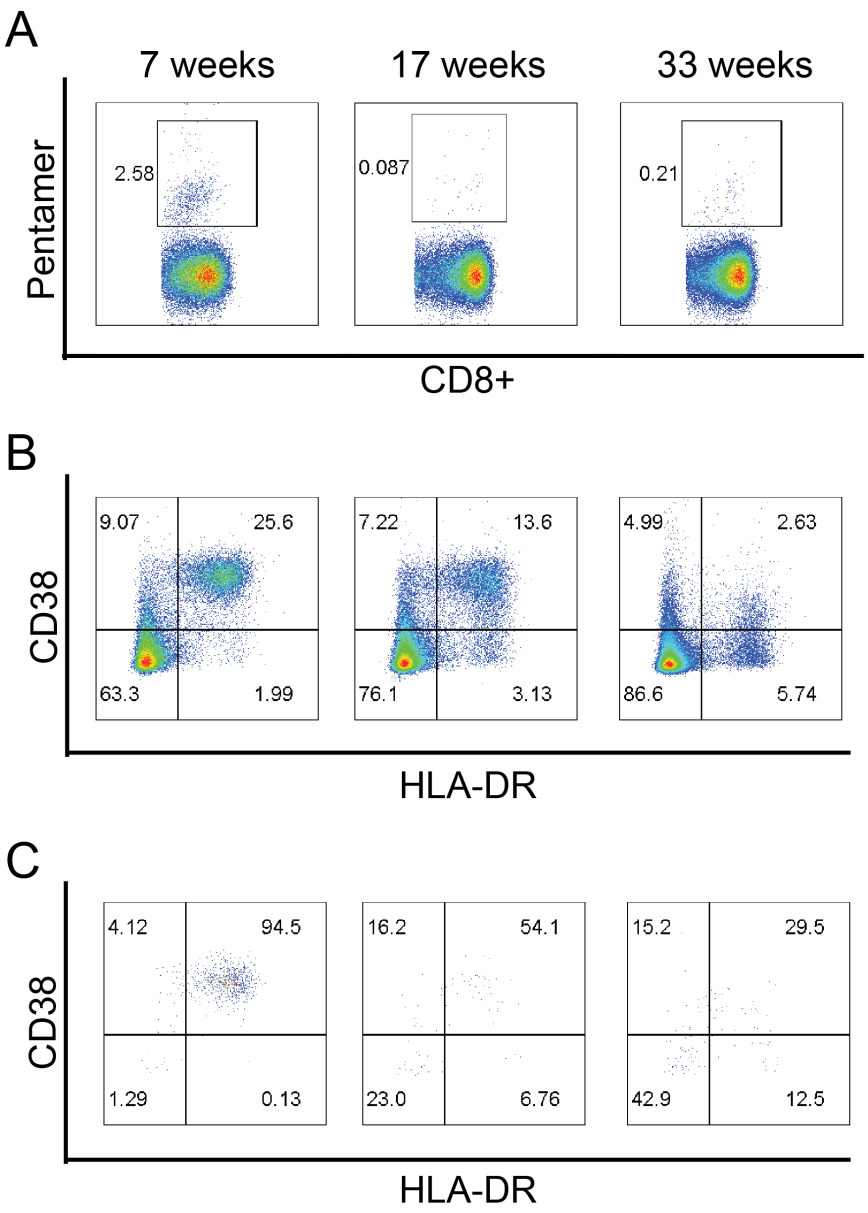
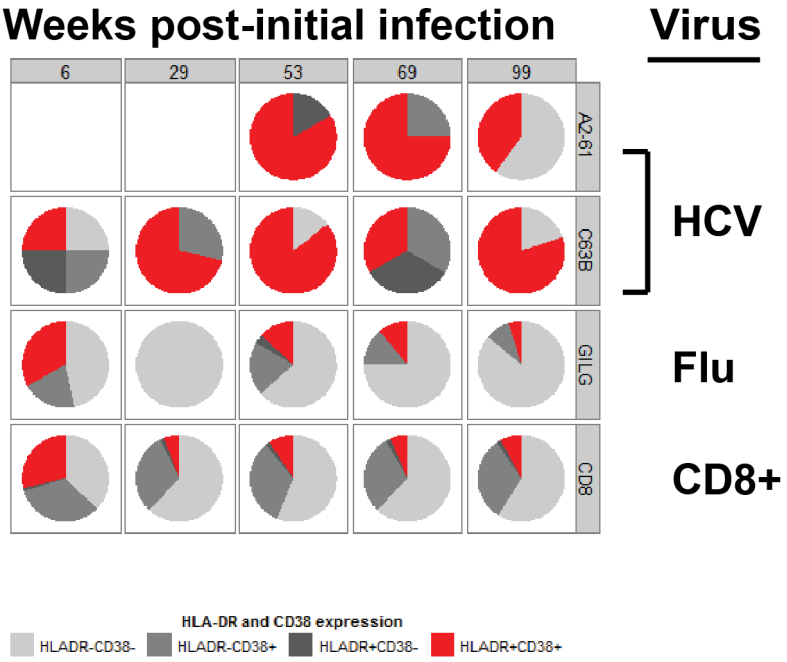


Figure 7 Representative flow cytometry staining of HLA-DR and CD38 expression

PBMCs from 10 HCV-infected subjects at multiple time points were stained and analyzed for CD3+CD8+ and antigen-specificity using pentamer technology. Cells were then analyzed for their expression of HLA-DR and CD38. Representative staining is shown for Subject 461. (A) Pentamer staining over 7, 17, and 33 weeks post infection. (B) CD38 and HLA-DR expression on the global CD8+ T cell population. (C) CD38 and HLA-DR expression on CD8+ T cells specific for the C63B HCV epitope (HLA A*02 NS3 1071-1081 CINGVCWTV).

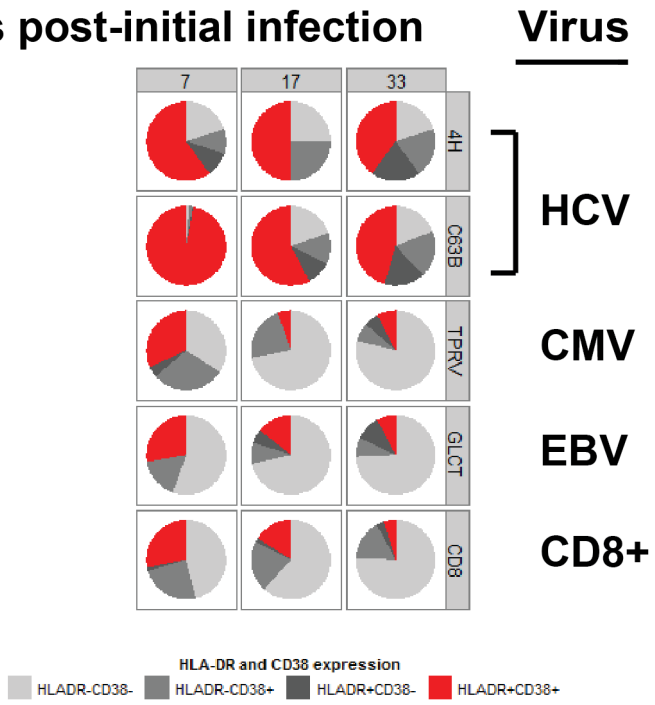
A

Subject 175



B

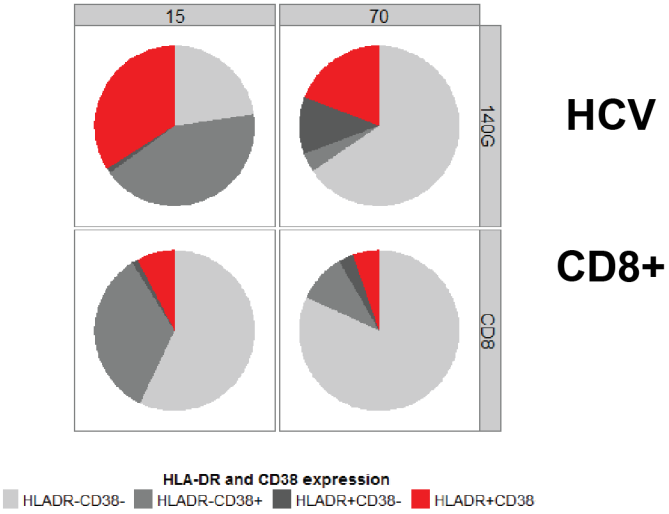
Subject 461



C

Subject 26

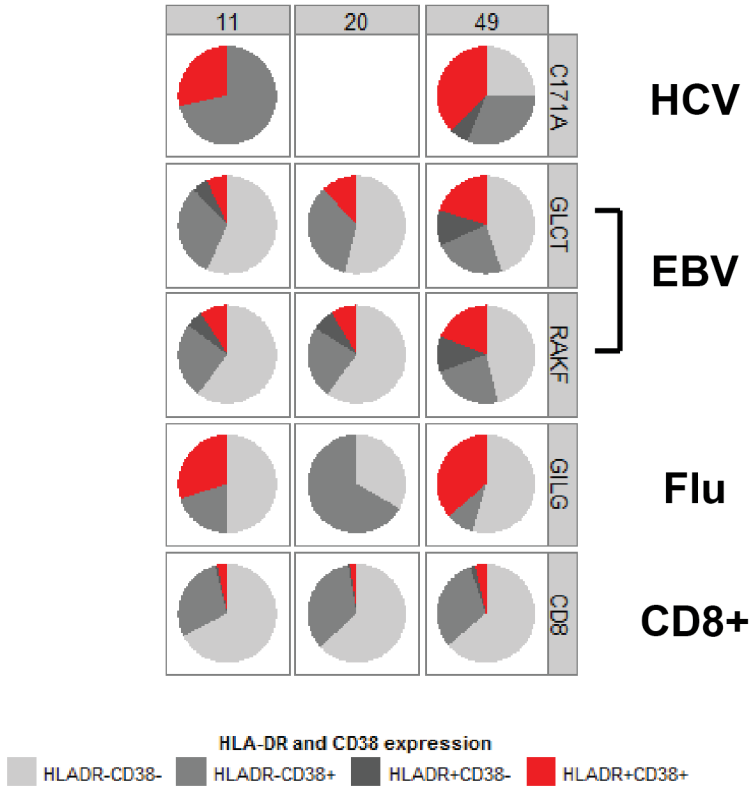
Weeks post-initial infection **Virus**



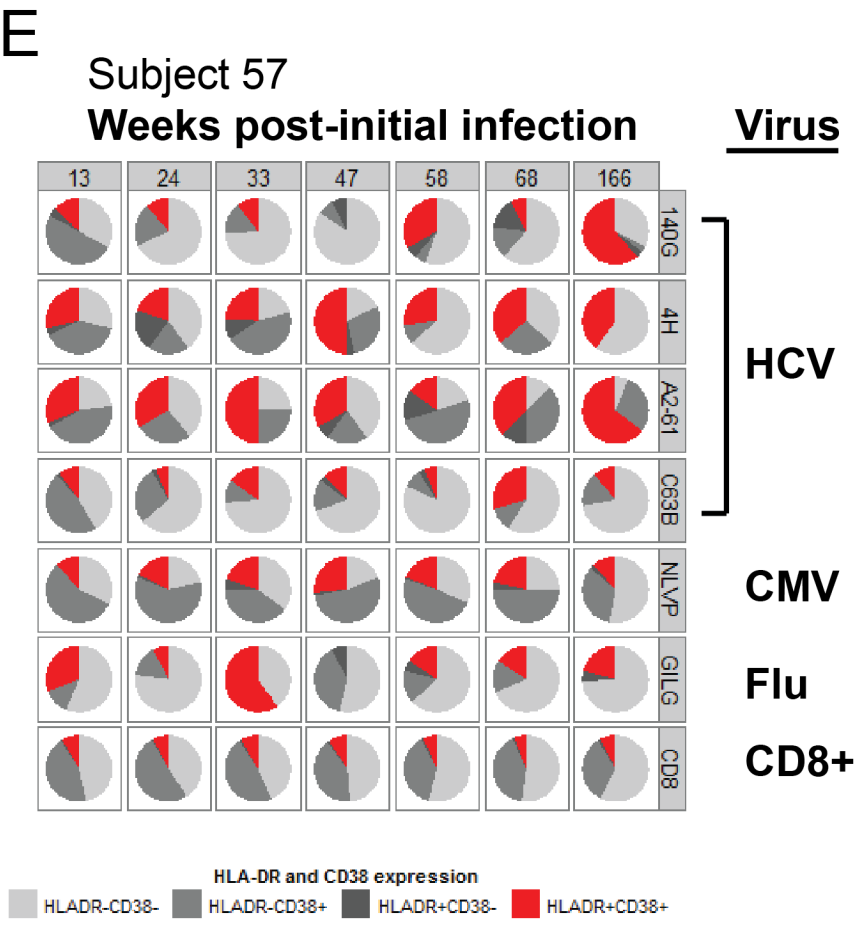
D

Subject 269

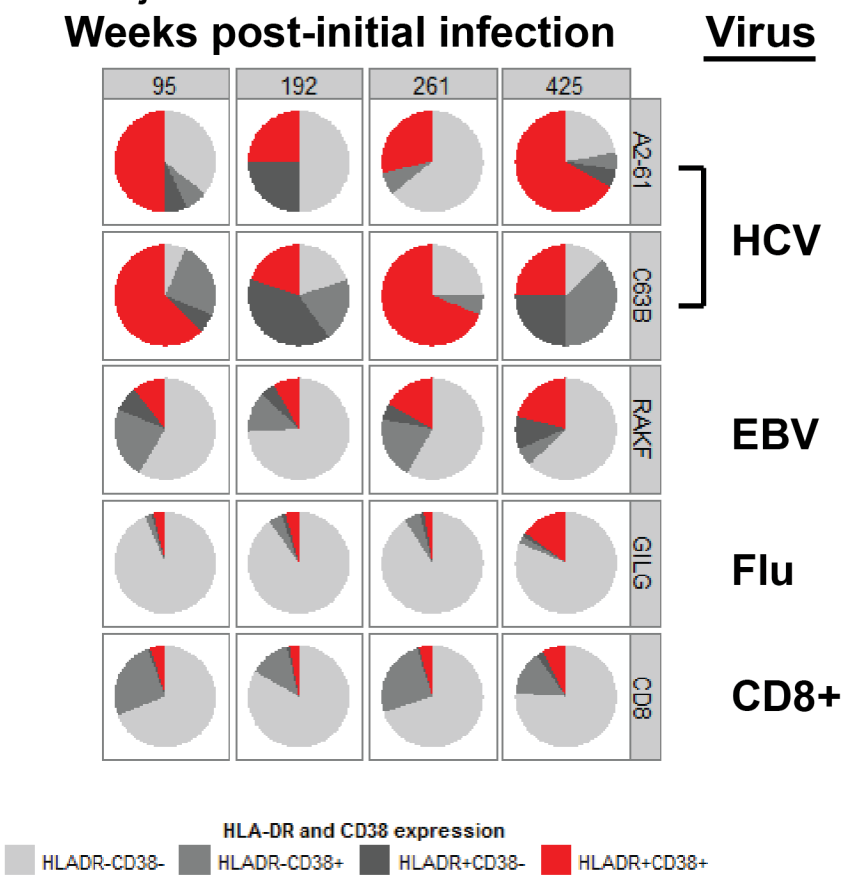
Weeks post-initial infection **Virus**



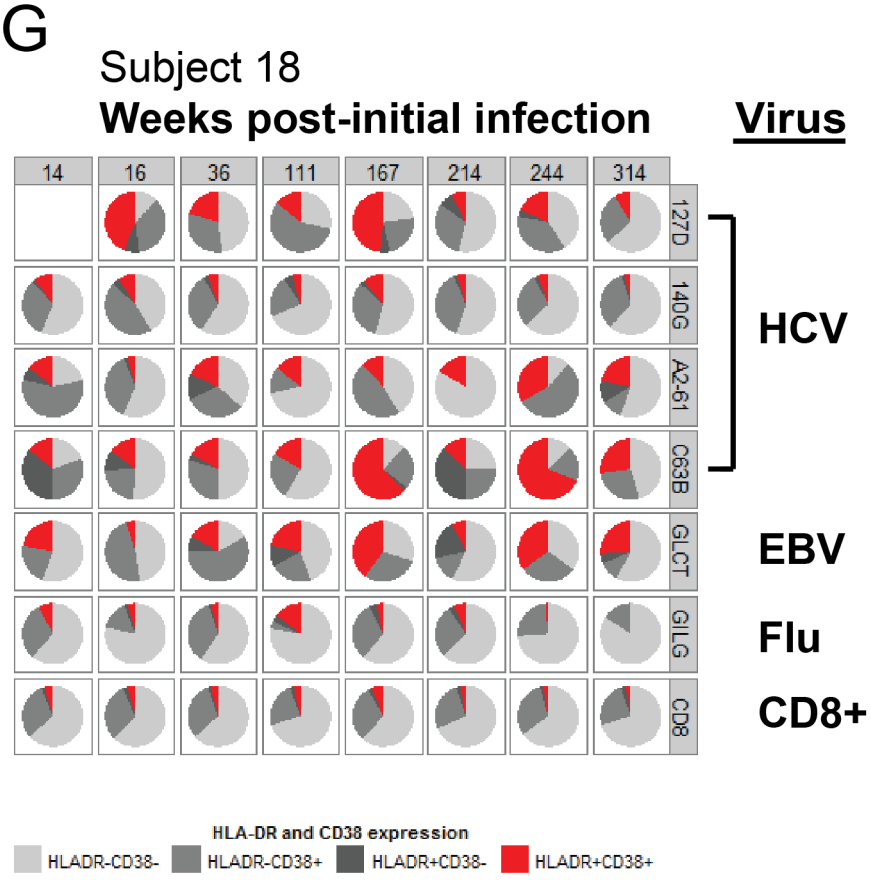
E


Subject 57
Weeks post-initial infection
Virus
13
24
33
47
58
68
166
140G
4H
A2-61
C63B
NLVP
GILG
CD8
HCV
CMV
Flu
CD8+
HLA-DR and CD38 expression
HLADR-CD38-
HLADR-CD38+
HLADR+CD38-
HLADR+CD38+

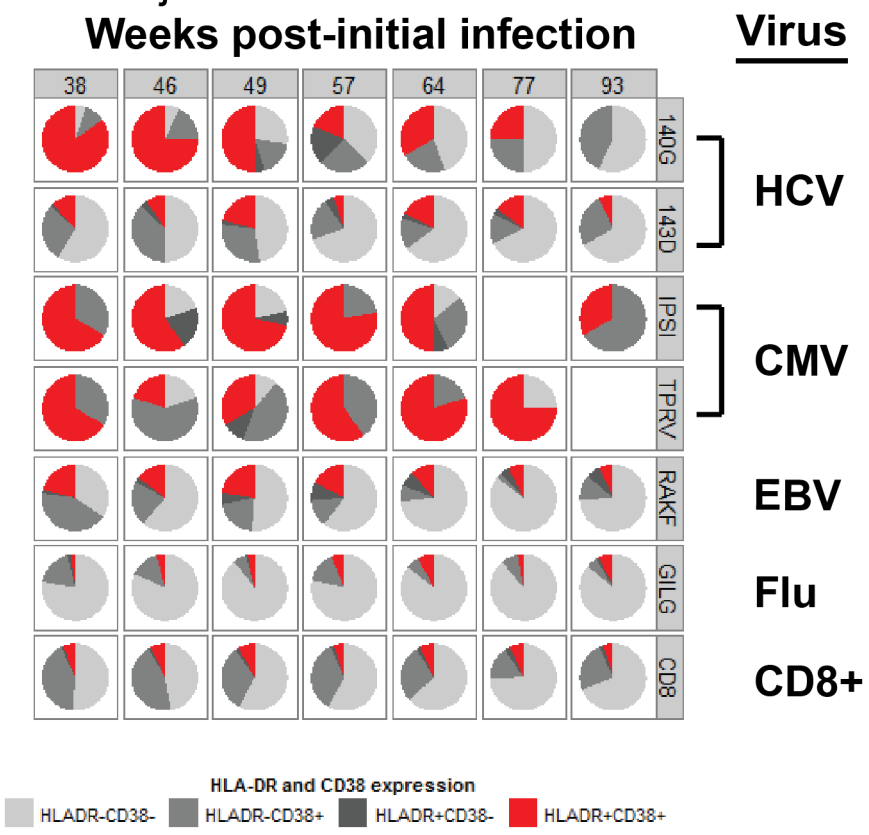
F


Subject 30
Weeks post-initial infection
Virus
95
192
261
425
A2-61
C63B
RAKF
GILG
CD8
HCV
EBV
Flu
CD8+
HLA-DR and CD38 expression
HLADR-CD38-
HLADR-CD38+
HLADR+CD38-
HLADR+CD38+

G


Subject 18
Weeks post-initial infection
Virus
14
16
36
111
167
214
244
314
127D
140G
A2-61
C63B
GLCT
GILG
CD8
HCV
EBV
Flu
CD8+
HLA-DR and CD38 expression
HLADR-CD38-
HLADR-CD38+
HLADR+CD38-
HLADR+CD38+

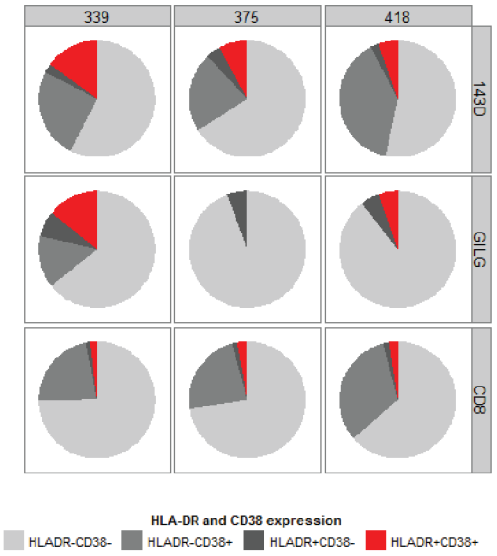
H


Subject 115
Weeks post-initial infection
Virus
38
46
49
57
64
77
93
140G
143D
IPSI
TPRV
RAKF
GILG
CD8
HCV
CMV
EBV
Flu
CD8+
HLA-DR and CD38 expression
HLADR-CD38-
HLADR-CD38+
HLADR+CD38-
HLADR+CD38+

I

Subject 65

Weeks post-initial infection **Virus**



J

Subject 111

Weeks post-initial infection **Virus**

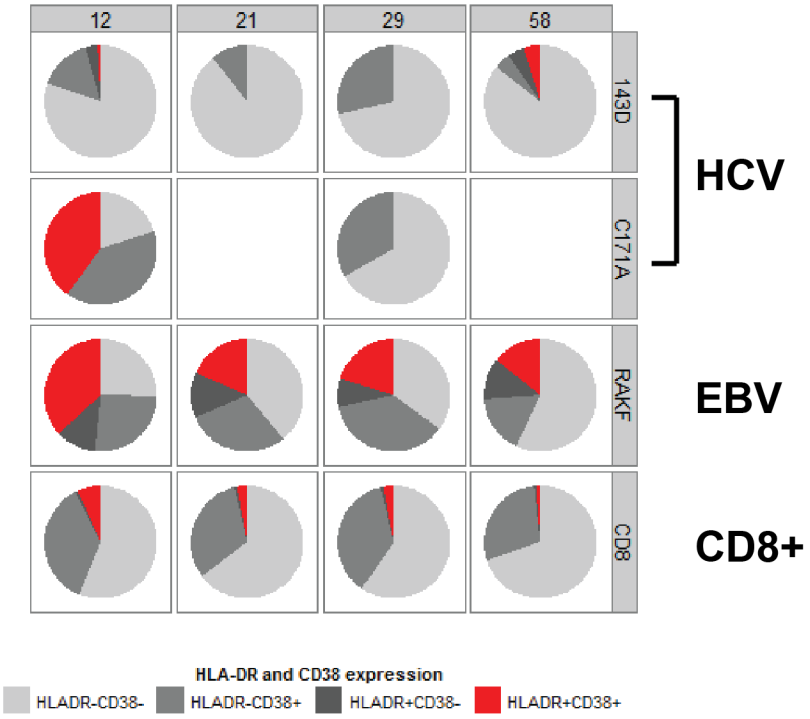
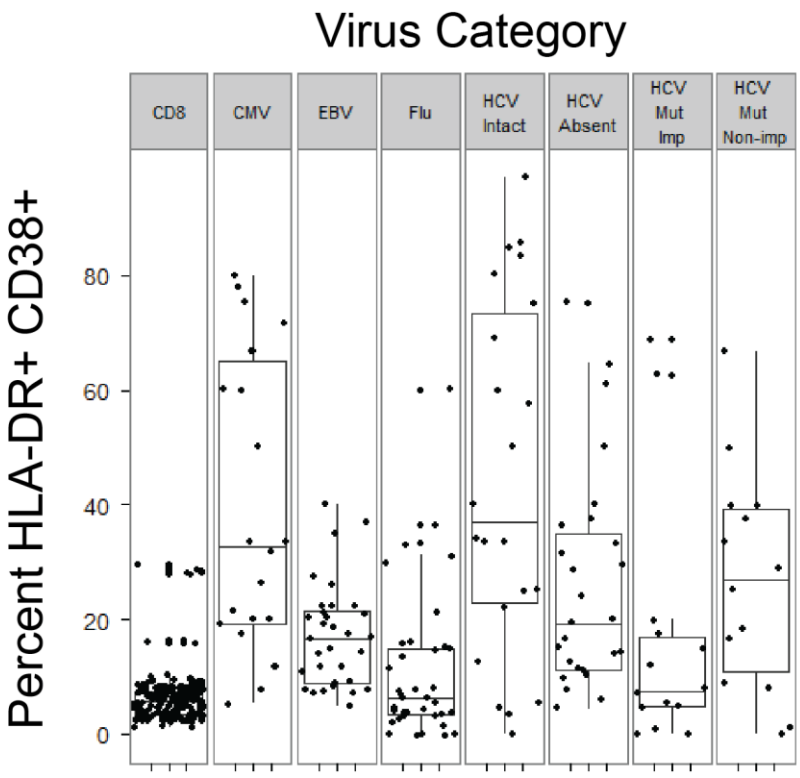


Figure 8 HLADR and CD38 are dually expressed throughout HCV infection

PBMCs from 10 HCV-infected subjects at multiple time points were stained and analyzed for CD3+CD8+ and antigen-specificity using pentamer technology. HCV, CMV, EBV, Flu-specific, and global CD8 T cells were then analyzed for expression of HLA-DR and CD38.(A-J) with the percent of single or double HLA-DR and CD38 expression shown and red shading indicating double expression. The horizontal axis indicates weeks post initial infection, and the vertical columns indicate the specific population of CD8+ T cells.

A


Virus Category
CD8
CMV
EBV
Flu
HCV Intact
HCV Absent
HCV Mut Imp
HCV Mut Non-imp
Percent HLA-DR+ CD38+
0
20
40
60
80

B

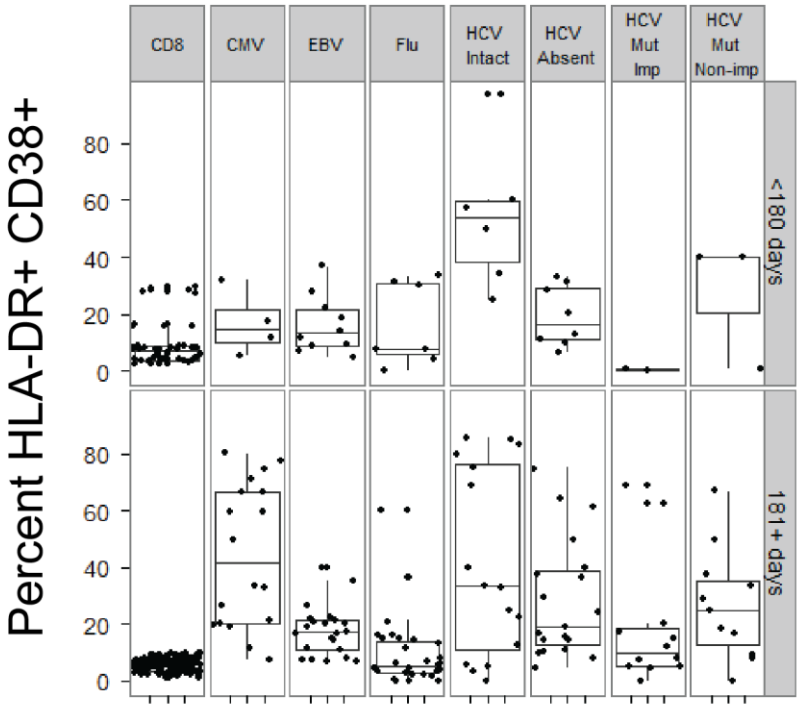

CD8
CMV
EBV
Flu
HCV Intact
HCV Absent
HCV Mut Imp
HCV Mut Non-imp
<180 days
181+ days
Percent HLA-DR+ CD38+
0
20
40
60
80

Figure 9 HLA-DR and CD38 are expressed on cells with intact circulating antigen.

PBMCs from 10 HCV-infected subjects at multiple time points were stained and analyzed for CD3+CD8+ and antigen-specificity using pentamer technology. Cells were then analyzed for their expression of HLA-DR and CD38. Samples were grouped in the following categories: global CD8+ population, the control viruses CMV, EBV, and influenza, and HCV. HCV-specific cells were further stratified based on whether the identified circulating virus was intact, absent, or contained mutations known to impair binding to the HLA (mut imp) or did not impair binding to the HLA (mut non-imp). (A) All samples irrespective of time of infection (B) Samples stratified prior to and after 180 days of infection.

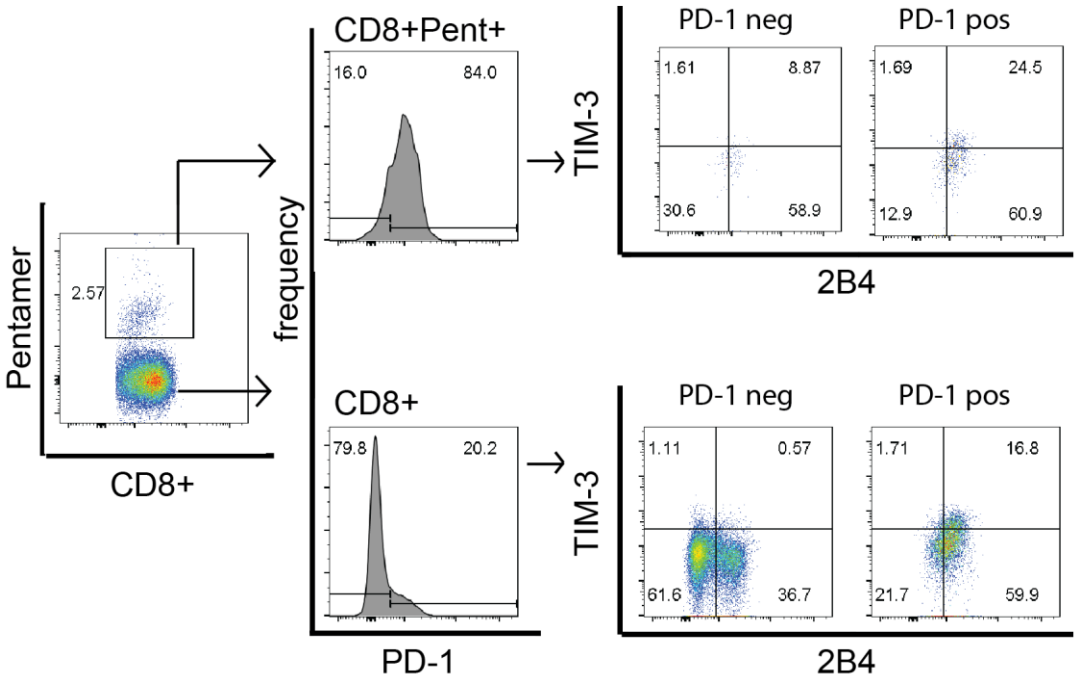
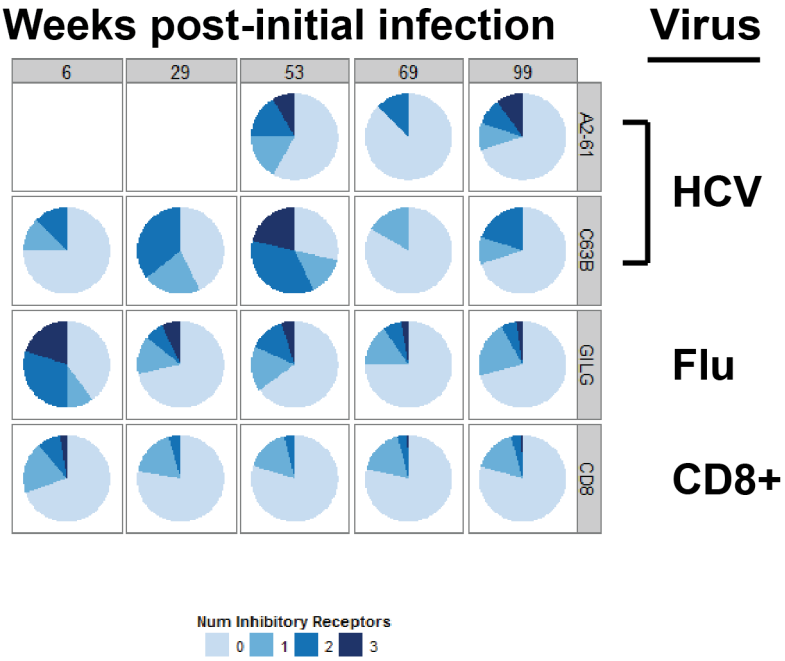


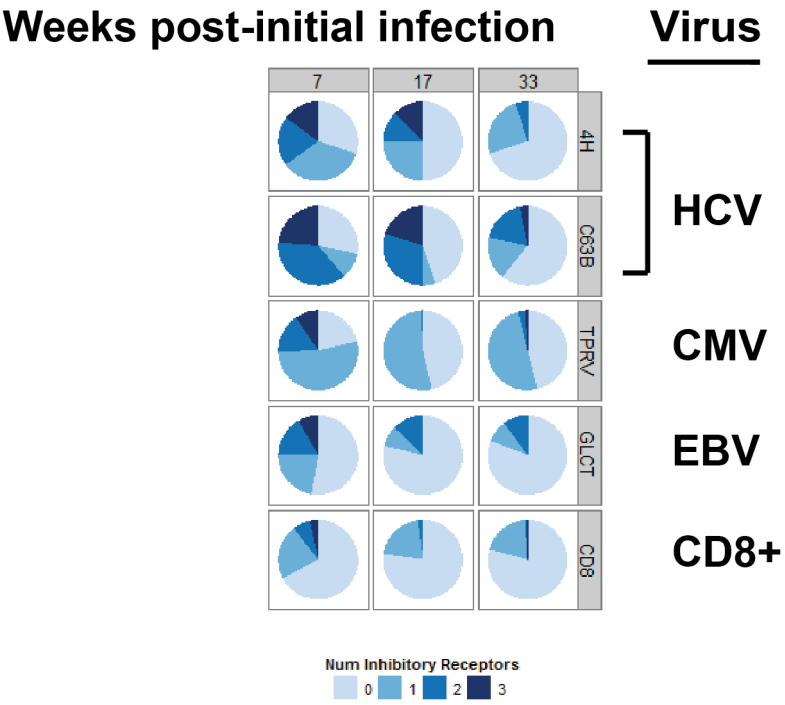
Figure 10 Representative FACS staining of PD-1, TIM-3, and 2B4

For Subject 461, PBMCs were stained and gated on CD3+CD8+ and antigen-specificity using pentamers. Cells were then analyzed for their expression of PD-1, TIM-3, and 2B4.

A


Subject 175
Weeks post-initial infection
Virus
6
29
53
69
99
A2-61
C63B
GILG
CD8
HCV
Flu
CD8+
Num Inhibitory Receptors
0 1 2 3

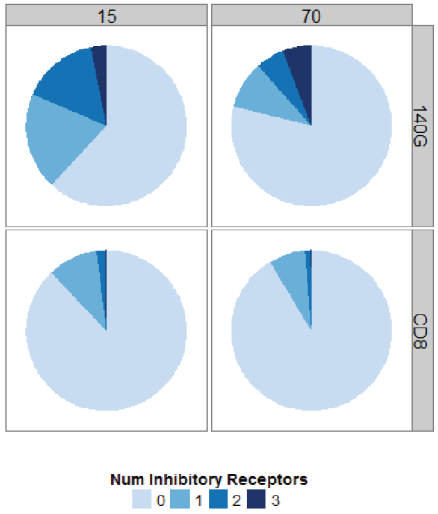
B


Subject 461
Weeks post-initial infection
Virus
7
17
33
4H
C63B
TPRV
GLCT
CD8
HCV
CMV
EBV
CD8+
Num Inhibitory Receptors
0 1 2 3

C

Subject 26

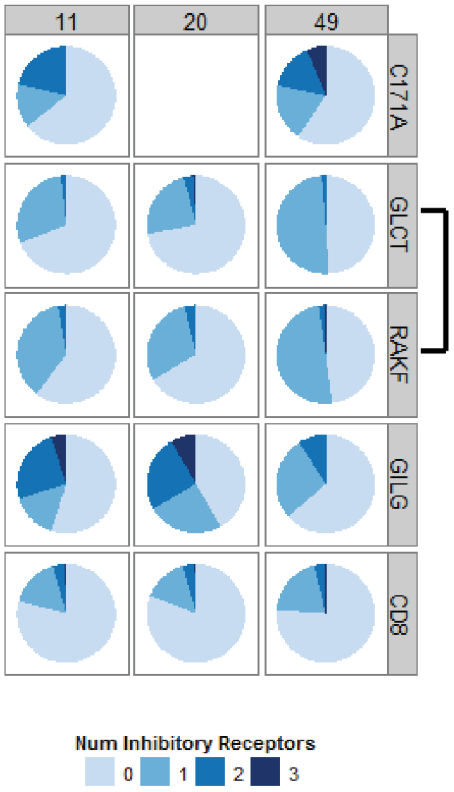
Weeks post-initial infection **Virus**



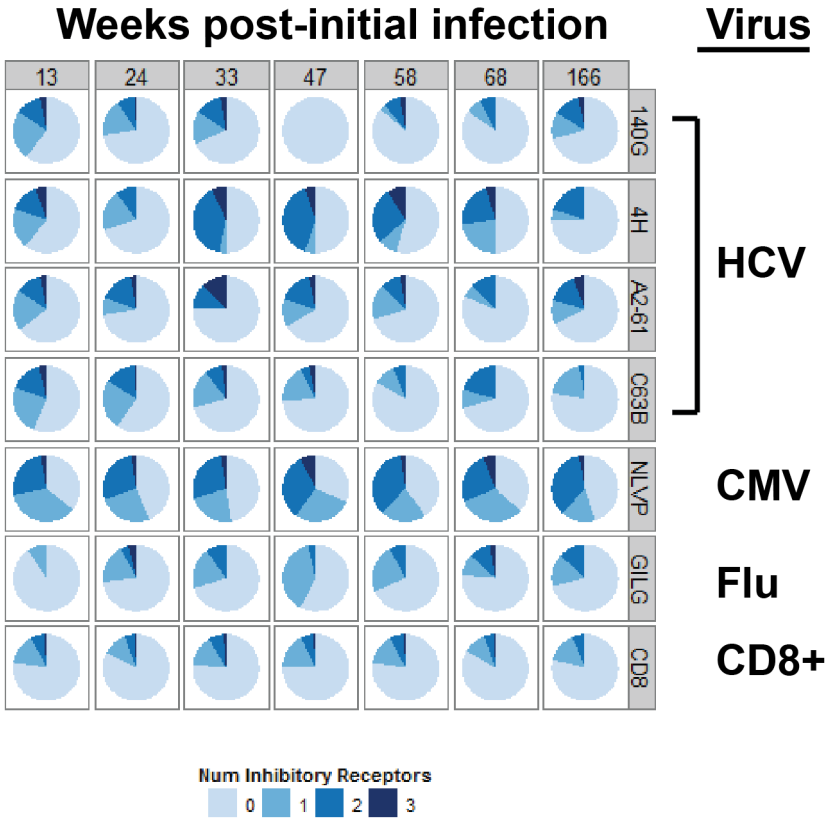
D

Subject 269

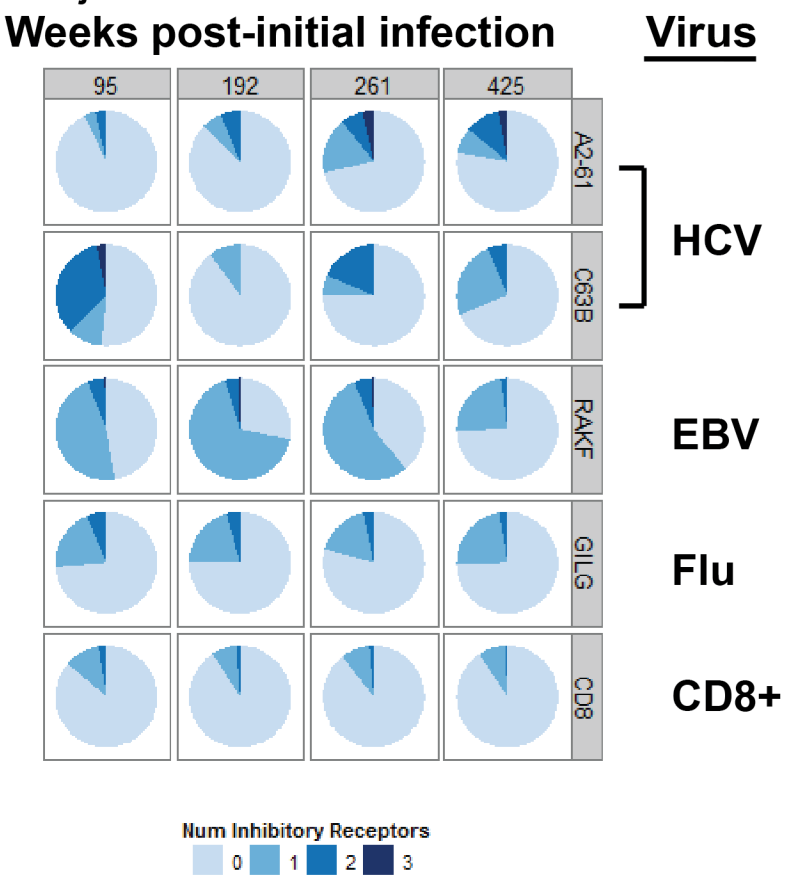
Weeks post-initial infection **Virus**



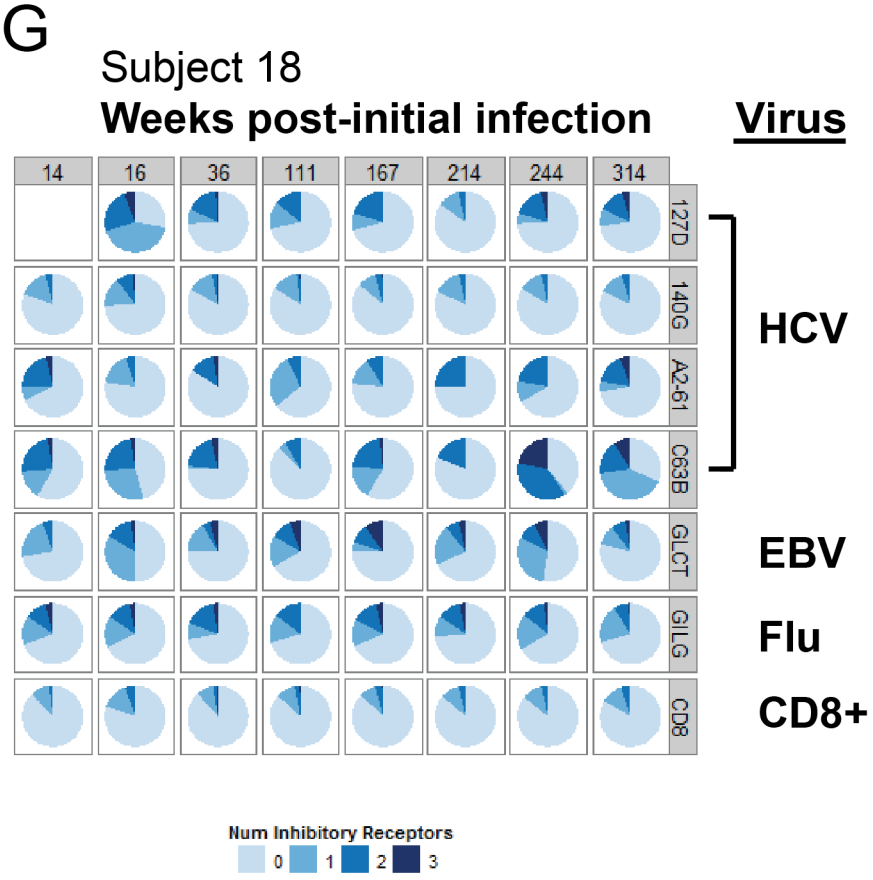
E


Subject 57
Weeks post-initial infection
Virus
13
24
33
47
58
68
166
140G
4H
A2-61
C63B
NLVP
GILG
CD8
HCV
CMV
Flu
CD8+
Num Inhibitory Receptors
0
1
2
3

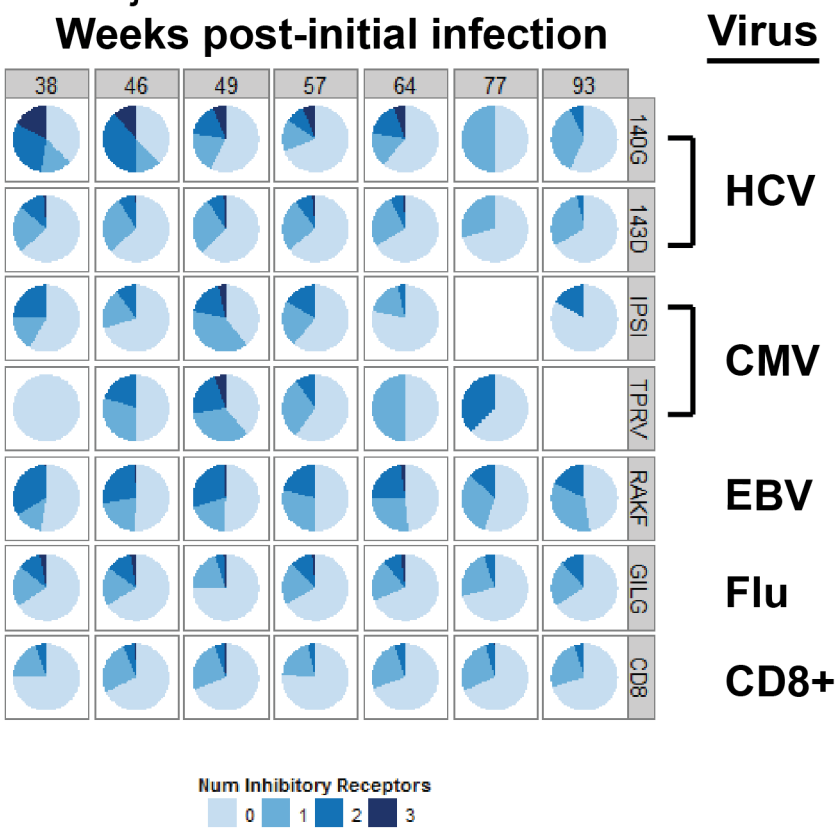
F


Subject 30
Weeks post-initial infection
Virus
95
192
261
425
A2-61
C63B
RAKF
GILG
CD8
HCV
EBV
Flu
CD8+
Num Inhibitory Receptors
0
1
2
3

G


Subject 18
Weeks post-initial infection
Virus
14
16
36
111
167
214
244
314
127D
140G
A2-61
C63B
GLCT
GILG
CD8
HCV
EBV
Flu
CD8+
Num Inhibitory Receptors
0
1
2
3

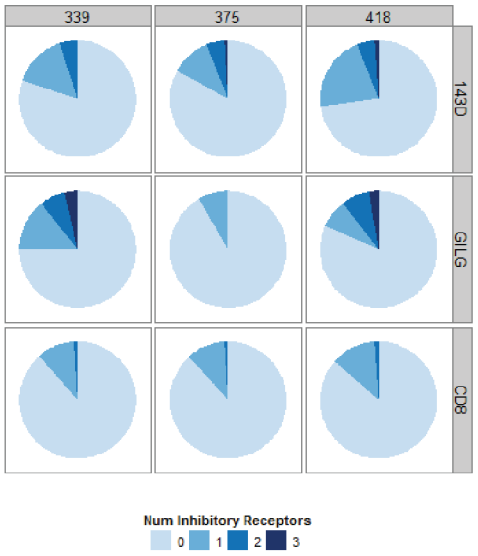
H


Subject 115
Weeks post-initial infection
Virus
38
46
49
57
64
77
93
140G
143D
IPSI
TPRV
RAKF
GILG
CD8
HCV
CMV
EBV
Flu
CD8+
Num Inhibitory Receptors
0
1
2
3

I

Subject 65

Weeks post-initial infection



Virus

HCV

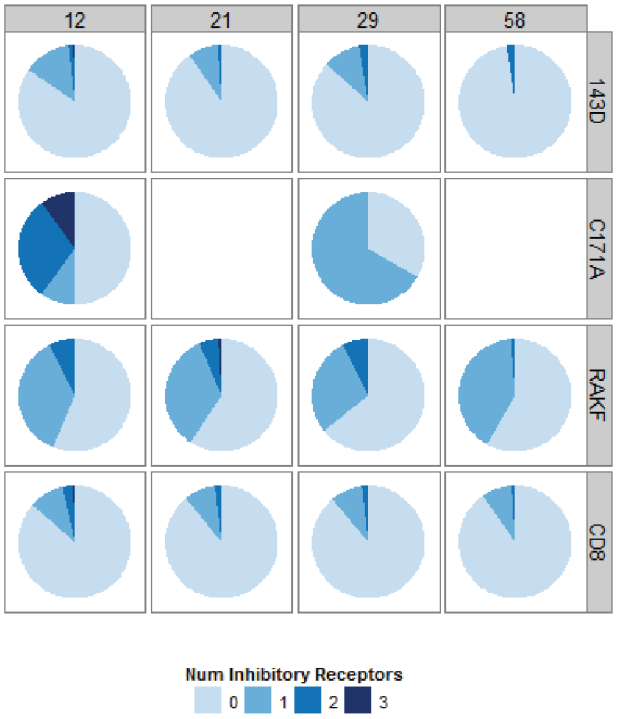
Flu

CD8+

J

Subject 111

Weeks post-initial infection



Virus

HCV

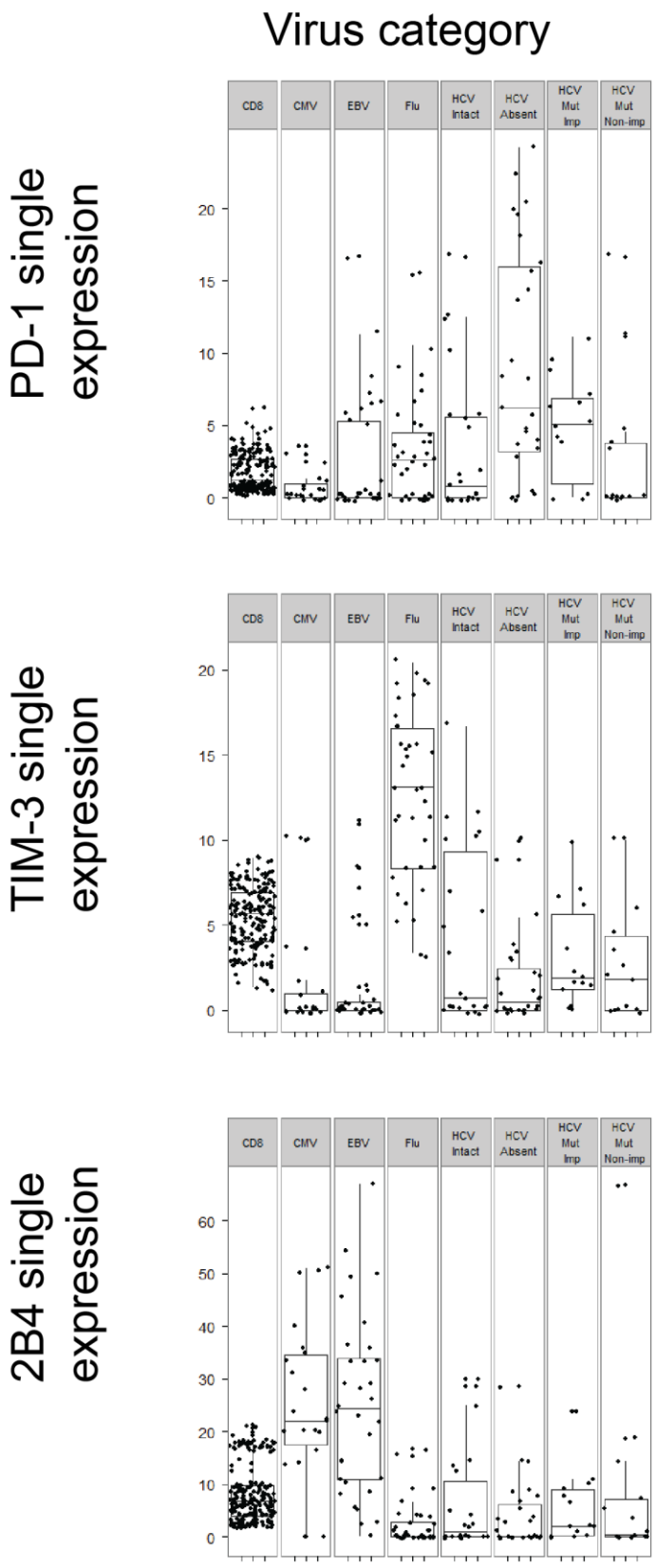
EBV

CD8+

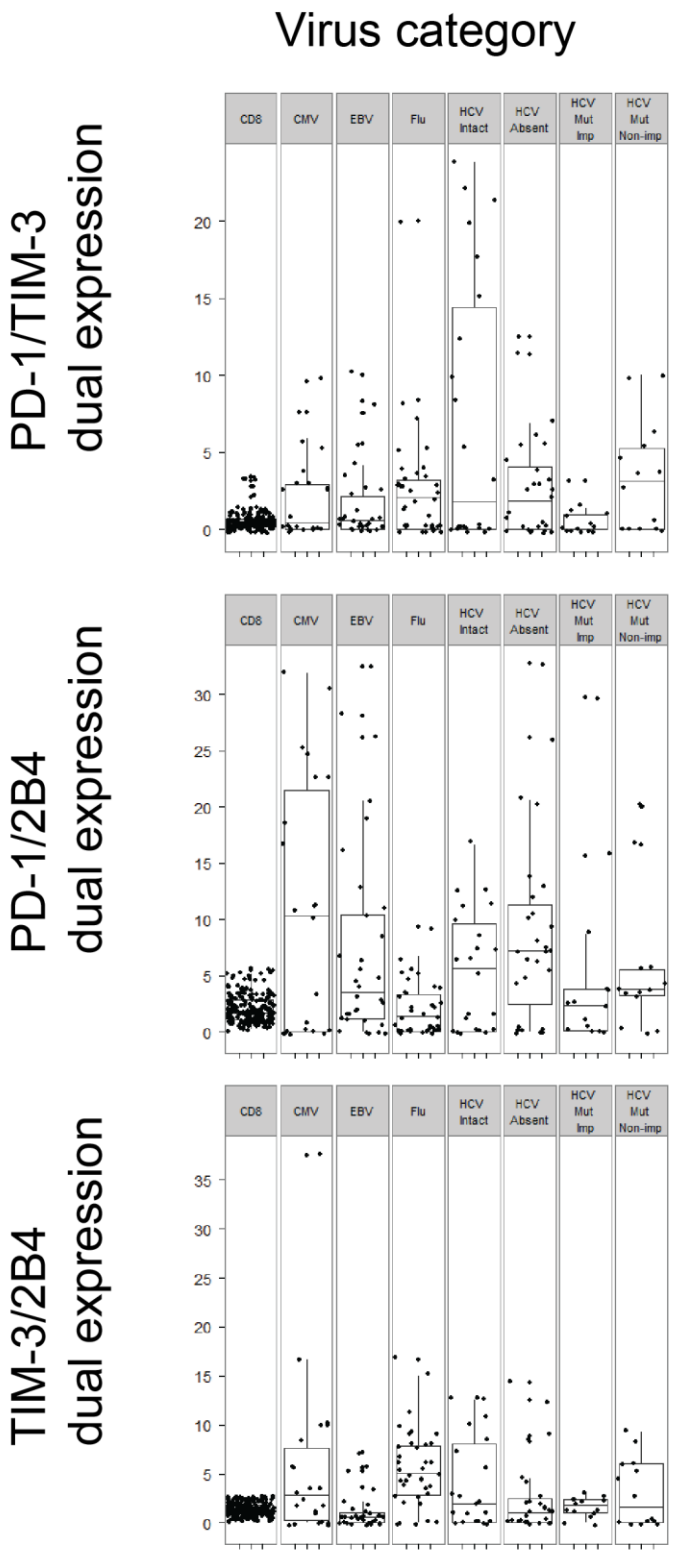
Figure 11 Expression of co-inhibitory molecules diminishes over time.

PBMCs from 10 HCV-infected subjects at multiple time points were stained and analyzed for CD3+CD8+ and antigen-specificity using pentamers. Cells were then analyzed for their expression of PD-1, TIM-3, and 2B4. The percentage of cells expressing zero, one, two ,or three inhibitory receptors (PD-1, TIM-3, 2B4) on HCV, CMV, EBV, Flu-specific, and global CD8 T cell population was calculated and expressed in a pie graph (A-J). The horizontal axis indicates weeks post initial infection, and the vertical columns indicate the specific population of CD8+ cells

A


Virus category
PD-1 single expression
TIM-3 single expression
2B4 single expression
CD8
CMV
EBV
Flu
HCV Intact
HCV Absent
HCV Mut Imp
HCV Mut Non-imp

B


Virus category
PD-1/TIM-3 dual expression
PD-1/2B4 dual expression
TIM-3/2B4 dual expression
CD8
CMV
EBV
Flu
HCV Intact
HCV Absent
HCV Mut Imp
HCV Mut Non-imp

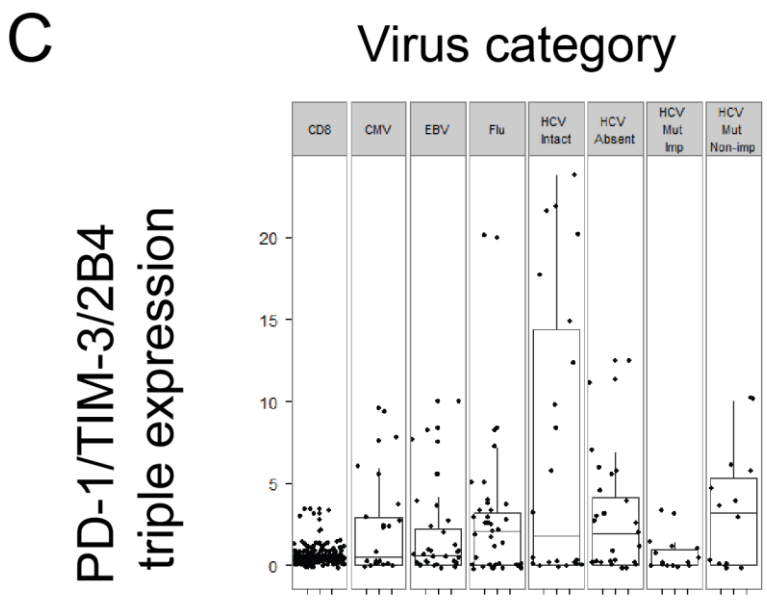


Figure 12 Expression of co-inhibitory molecules differ based on circulating virus sequence.

PBMCs from 10 HCV-infected subjects at multiple time points were stained and analyzed for CD3+CD8+ and antigen-specificity using pentamers. Cells were then analyzed for their expression of PD-1, TIM-3, and 2B4.. Samples were grouped in the following categories: global CD8+ population, the control viruses CMV, EBV, and influenza, and HCV. HCV-specific cells were further stratified based on whether the identified circulating virus was intact, absent, or contained mutations known to impair binding to the HLA (mut imp) or did not impair binding to the HLA (mut non-imp). (A) Percentage of cells with single expression of only PD-1, TIM-3, or 2B4. (B). Percentage of cells with dual expression patterns of PD-1/TIM-3, PD-1/2B4, and TIM-3/2B4. (C) Percentage of cells with triple expression of PD-1, TIM-3, and 2B4.

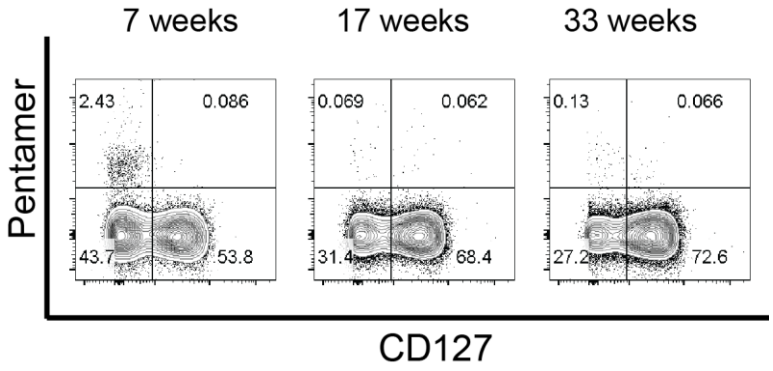


Figure 13 Representative FACS staining of CD127

Representative FACS staining of CD127 on Subject 461 for weeks 7, 17, and 33. PBMCs were stained and gated on CD3+CD8+ and antigen-specificity using pentamer technology. Cells were then analyzed for their expression of CD127.

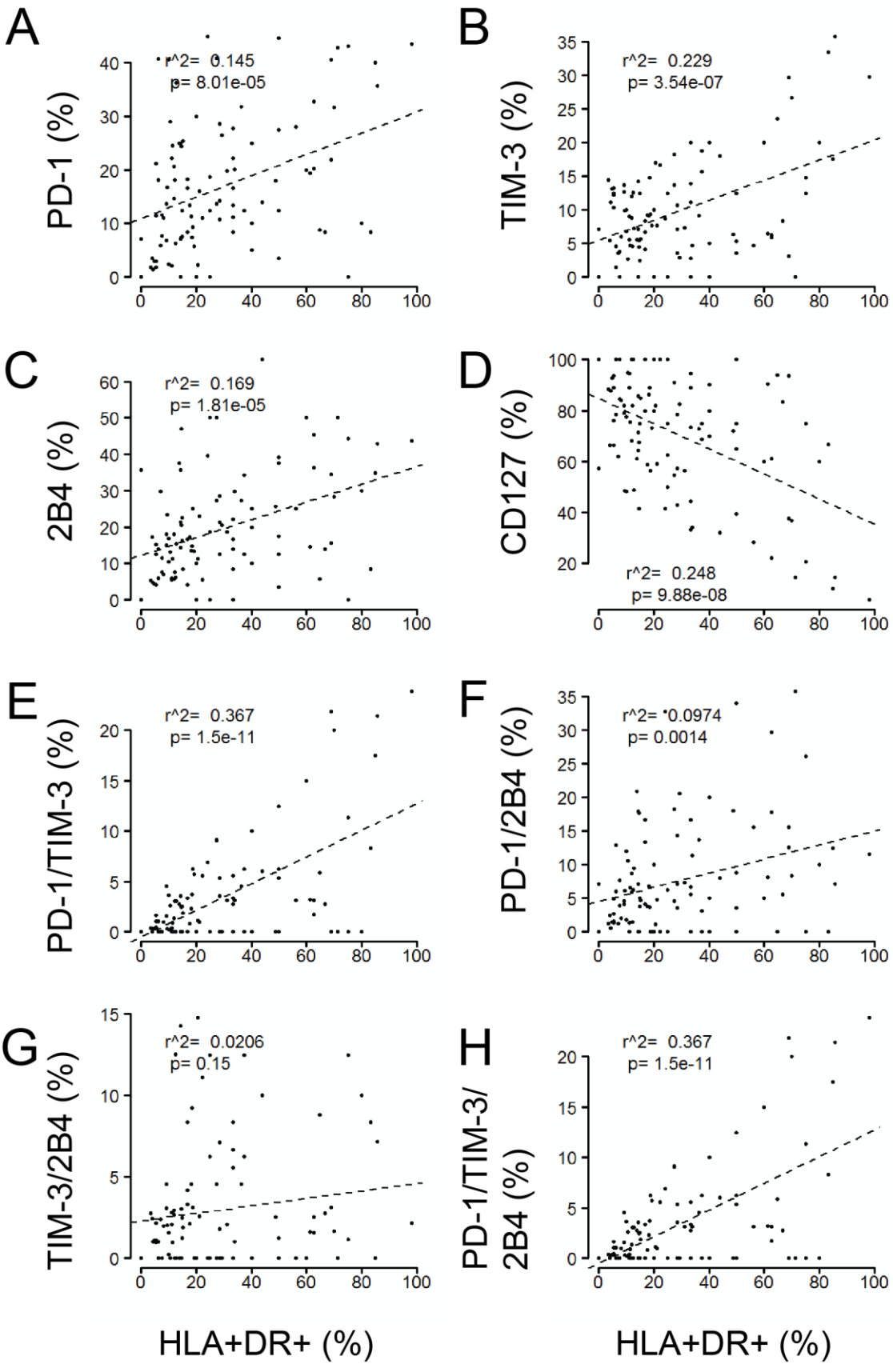
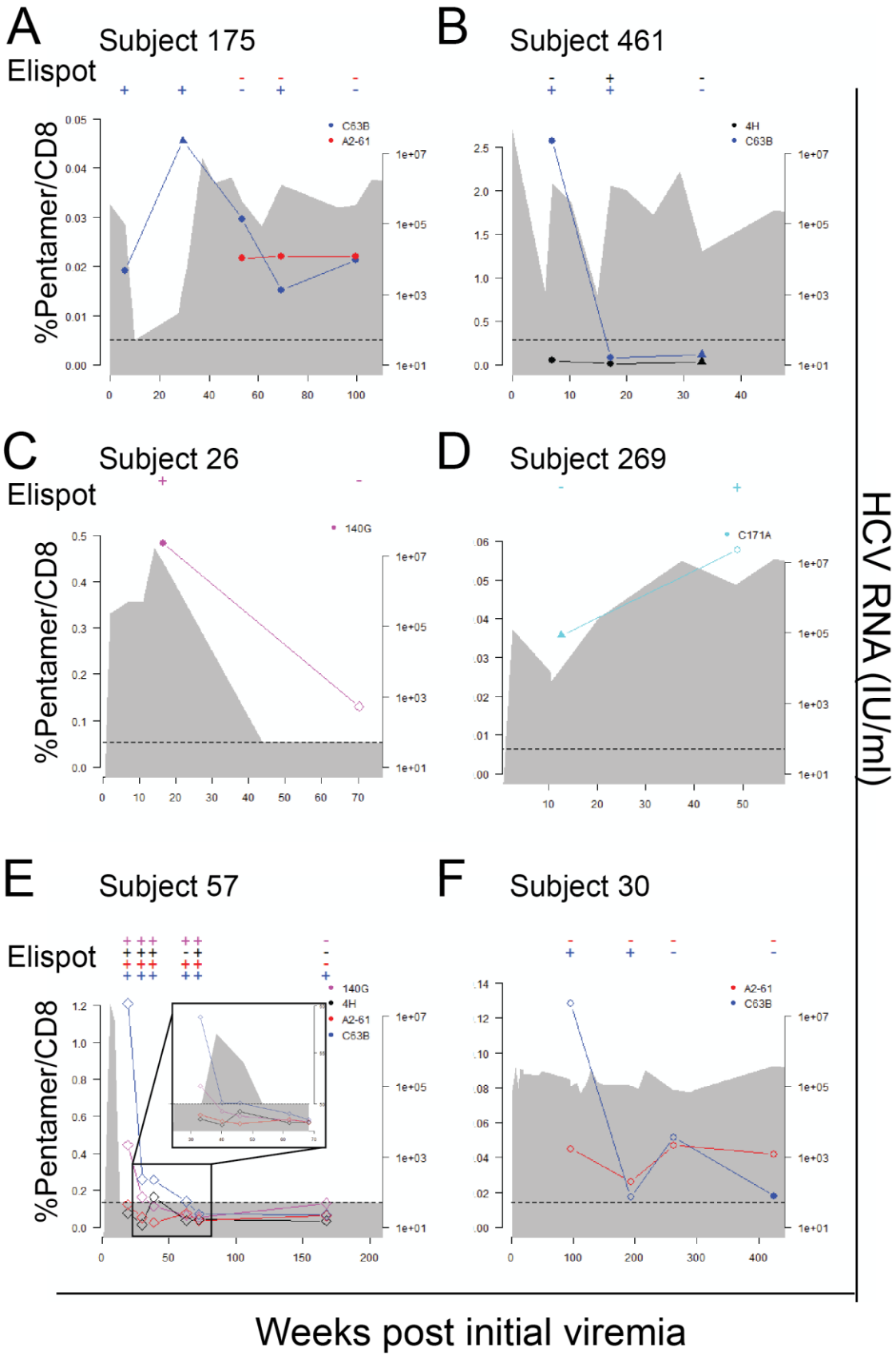

A
PD-1 (%)
r^2= 0.145
p= 8.01e-05
B
TIM-3 (%)
r^2= 0.229
p= 3.54e-07
C
2B4 (%)
r^2= 0.169
p= 1.81e-05
D
CD127 (%)
r^2= 0.248
p= 9.88e-08
E
PD-1/TIM-3 (%)
r^2= 0.367
p= 1.5e-11
F
PD-1/2B4 (%)
r^2= 0.0974
p= 0.0014
G
TIM-3/2B4 (%)
r^2= 0.0206
p= 0.15
H
PD-1/TIM-3/ 2B4 (%)
r^2= 0.367
p= 1.5e-11
HLA+DR+ (%)
HLA+DR+ (%)

Figure 14 Triple expression of co-inhibitory molecules is strong positively correlated with HLA-DRA and CD38 dual expression on HCV-specific CD8+ T cells

Cells were analyzed by flow cytometry for expression of co-inhibitory molecules PD-1, TIM-3, and 2B4, the differentiation molecule CD127, and the activation markers HLA-DR and CD38. Patterns of inhibitory receptor and CD127 expression HLA-DR+ CD38+dual expression (x-axis) versus CD127 or co-inhibitory receptor (y-axis). (A) PD-1 (B) TIM-3 (C) 2B4 (D) CD127 (E) PD-1 and TIM-3 (F) PD-1 and 2B4 (G) TIM-3 and 2B4 and (H) PD-1, TIM-3, and 2B4


A
Subject 175
Elispot
%Pentamer/CD8
C63B
A2-61
B
Subject 461
4H
C63B
C
Subject 26
140G
D
Subject 269
C171A
E
Subject 57
140G
4H
A2-61
C63B
F
Subject 30
A2-61
C63B
HCV RNA (IU/ml)
Weeks post initial viremia

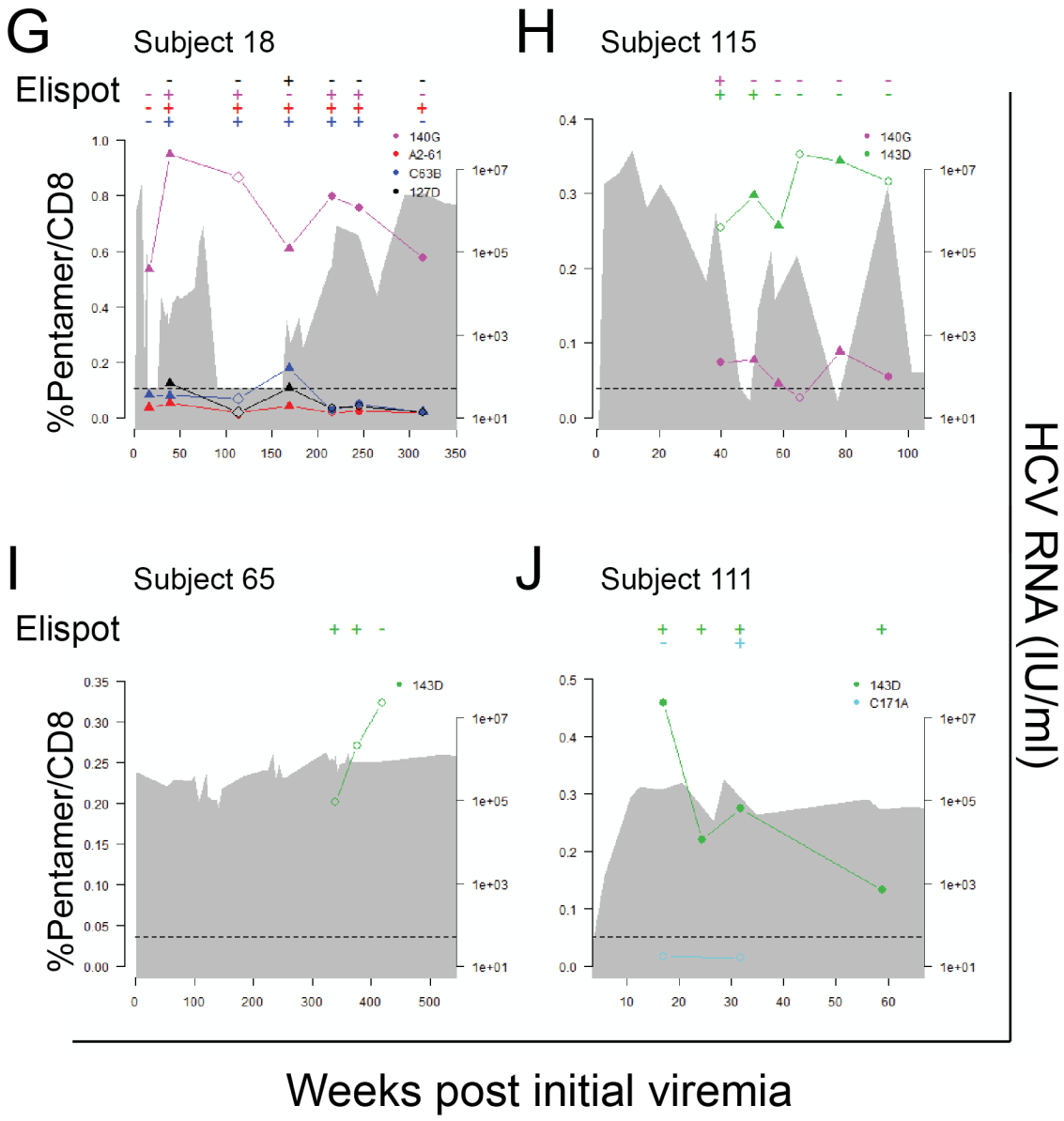


Figure 15 Antigen-specific cells are detectable after functional response is not detectable. Shaded region indicates RNA viremia (IU/ml, right y-axis). Line graphs indicate percent of pentamer-specific events per CD8+ population (left y-axis) versus weeks post initial viremia. Symbols indicate viral sequence intact (-●-), viral sequence with substitutions relative to initial viremia (-○-), viral sequence absent (-◊-), or unable to be sequenced (-∆-). At these time points, sequences were also tested for IFN-γ response by ELISpot

with positive (+) or negative (-) responses indicated at the given time point. Colors indicate epitope tested.

Reference List

1. World Health Organization. 1997. Hepatitis C: global prevalence. *Weekly Epidemiological Record* 72: 341-348.

2. Wong, J. B., G. M. McQuillan, J. G. McHutchison, and T. Poynard. 2000. Estimating future hepatitis C morbidity, mortality, and costs in the United States. *Am. J. Public Health* 90: 1562-1569.

3. Alter, M. J., D. Kruszon-Moran, O. V. Nainan, G. M. McQuillan, F. Gao, L. A. Moyer, R. A. Kaslow, and H. S. Margolis. 1999. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. *N. Engl. J. Med.* 341: 556-562.

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Kelly Burke
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Curriculum Vitae

Kelly P. Burke
Date of Birth: November 3, 1983 Hometown: Woodbury, MN
Viral Hepatitis Center, Rangos Building 530, 855 North Wolfe St, Baltimore MD 21205
Cell phone: (651-249-3136)
kelly.burke@jhmi.edu

Education

Graduate:
MD/PhD, Johns Hopkins School of Medicine, Baltimore, MD May 2014

Undergraduate:
B.A. in Chemistry, *summa cum laude*, Kenyon College, Gambier, OH 2006

Public health study abroad in Port Elizabeth, South Africa (no degree) 2004
School for International Training, Brattleboro, VT

Research Experience

Johns Hopkins School of Medicine, Baltimore MD 2008 – 2014
Graduate researcher, Immunology program; Advisor: Andrea Cox, MD, PhD

- Dissertation: Interrogating the human CD8+ T cell response in HCV infection

Kenyon College, Gambier OH 2003 – 2006
Summer Science Scholar and undergrad researcher; Advisor: Mo Hunsen, PhD

- Synthesized secondary amines using carbohydrates as protecting groups, as characterized by 1D and 2D nuclear magnetic resonance

Washington University in St. Louis, St. Louis, MO Summer 2005
Biomedical Research Apprentice; Advisor: Shrikant Anant, PhD

- Investigated the transcriptional and post-transcriptional regulation of pro-inflammatory compounds by gastrin in a human gastric cancer cell line
- Utilized molecular and biochemical techniques, including real-time PCR

Mayo Clinic, Rochester, MN Summer 2004
Summer Undergraduate Research Fellowship; Advisor: Larry Karnitz, PhD

- Investigated effects of DNA damage checkpoint kinase inhibitor on the cell cycle
- Presented at departmental seminar; results comprised pre-clinical study report to pharmaceutical company

Publications: Peer Reviewed Scientific Articles

S. Munshaw, Bailey JR, Liu L, Osburn WO, **Burke KP,** Cox AL, and Ray SC. Computational reconstruction of Bole1a, a representative synthetic hepatitis C virus subtype 1a genome. *J Virol*. 2012; 86: 5915-5921.

Burke KP, Munshaw S, Osburn WO, Levine J, Liu L, Sidney J, Sette A, Ray SC, and Cox AL. Immunogenicity and cross-reactivity of a representative ancestral sequence in hepatitis C virus infection. *J Immunology*. 2012; 188: 5177-5188.

Publications: Reviews

Burke KP and Cox AL. Hepatitis C virus evasion of adaptive immune responses: a model for viral persistence. *Immun Research*.2008; 47: 216-227. Review.

Abstracts

Burke KP, Osburn WO, Levine J, Munshaw S, Liu L, Sidney J, Sette A, Ray SC, and Cox AL. "Biologic evidence of HCV ancestral sequence immunogenicity." Keystone Symposium. February 7, 2011. Poster presentation.

Burke KP, Munshaw S, Osburn WO, Levine J, Liu L, Ray SC, and Cox AL. HCV genotype 1a ancestral sequence elicits broad CD8+ T cell responses. 18th International Symposium on Hepatitis C Virus and Related Viruses. September 9, 2011. Oral presentation.

Burke KP, Munshaw S, Osburn WO, Levine J, Liu L, Ray SC, and Cox AL. Immunogenicity of a rationally designed hepatitis C virus sequence. Immunology 2012. May 6, 2012. Poster presentation.

Awards

Undergraduate:
Phi Beta Kappa and Sigma Xi
American Chemical Society, Division of Polymer Sciences Award
Barry M. Goldwater Scholarship
Elmer Graham Scholarship
Kenyon College Honor Science Scholarship
National Merit Scholarship
Robert C. Byrd Scholarship
Tozer Scholarship

Student Activities

Creative writing tutor, Christopher's Place 2006 - 2008

- Taught weekly creative writing classes to men earning their GED
- Designed and implemented lesson plans: discussion of literary excerpt followed by creative writing and group sharing/feedback.
- Focused on connection between literature and the gentlemen's own experiences

Co-chair, Revisit Committee Spring, 2007

- Coordinated faculty talks, housing tours, and evening activities for prospective medical students

Peer Advisor 2008 - 2011

- Met individually and collectively with five medical students for three years

- Advised students on classwork, clinical rotations, and research opportunities

Medical School Admissions Interviewer 2009 - 2010

- Interviewed M.D. applicants to Johns Hopkins Medical School for 4 hrs/week for 6.5 months
- Reviewed half of all applications for interviewees

Chair, Ambassadors committee; MSTP Student Advisory Board Spring 2012-2013

- Acted as and recruited students to serve as tour guides, dinner coordinators, revisit panelists
- Initiated matching program for accepted students to hear from current students with similar interests

MSTP Student Advisor Spring 2012-present

- Meet collectively with three MD/PhD students
- Organize events in conjunction with faculty advisor